Bacteriological studies of trickling filters.

Halls, N. A.

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BACTERIOLOGICAL STUDIES OF TRICKLING FILTERS

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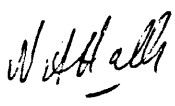

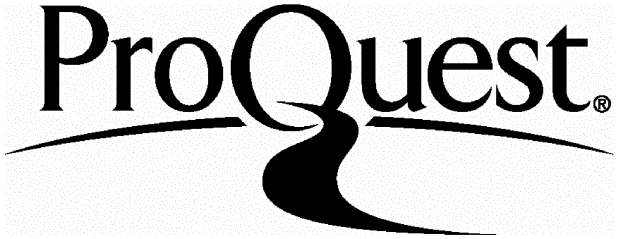

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SUMMARY

These studies were concerned with the microbial ecology of trickling filters with particular reference to their possible application in the disposal of wastes from intensively housed pigs. As it is technically difficult to obtain satisfactory samples from full-scale filter beds an apparatus similar to that of Gloyna, Comstock & Renn (1952) was used. Perspex tubes (2 ft x $2\frac{1}{2}$ in) were rested on rollers rotating at 18 rev/min. The chassis was inclined (1:25) and the wastes applied to the raised ends of the tubes.

Increase in weight was used as an index of the rate and extent of development of microbial film on the tubes and Chemical Oxygen Demand as an index of their efficiency of purification of wastes. When irrigated with domestic sewage the performance of the experimental filters was comparable with that reported in the literature for plastic-packed filters irrigated at comparable rates. Irrigation with piggery wastes, either neat or diluted, tended to inhibit purification and/or cause the film to slough. It was concluded that trickling filters were unlikely to be suitable for purification of wastes from intensively housed pigs unless the concentration of organic matter was reduced by dilution or prior treatment such as anaerobic digestion.

The microflora of films irrigated with domestic sewage was dominated by Acinetobacter and yellow-pigmented non-motile Gram-negative rod-shaped organisms. Irrigation with piggery wastes yielded a film consisting of Acinetobacter only. It was deduced that purification in certain aerobic waste treatment processes may be dependant upon the association of these two organisms. There is evidence from the literature to suggest that Pseudomonas may be dominant in other situations. The nature of the waste and the operational procedures of the plant apparently

having a selective influence on the microbial association which becomes dominant in any particular waste treatment process.

CONTENTS

INTRODUCTION

INTRODUCTION

Although the principal methods for the aerobic biological treatment of wastes, trickling filters and the activated sludge process, have been extensively used for many years and numerous technical modifications have been described information on the nature and composition of their microflora is relatively scarce. Virtually all sewage purification plants in the United Kingdom use some form of these processes. The majority of plants serving populations of less than 100,000 use trickling filters; at very large plants the activated sludge process is predominant while for populations between 100,000 and 500,000 the alternative systems are used to about the same extent (Bruce, 1969). For complete or partial oxidation of industrial wastes the more compact activated sludge process is commoner than filter beds (Ainsworth, 1966). However, the introduction of geometrically ordered plastic filter media for use in tall towers (Eden Truesdale & Mann, 1966) is making trickling filters more competitive because of their low operating costs, relatively simple operation and low susceptibility to sudden and perhaps inimical changes in the composition of the waste (Ainsworth, 1966). Now that the Public Health Act, 1961 (Anon, 1961a) has brought farm effluents in the United Kingdom under the surveillance of pollution prevention authorities, waste from pig production units must be disposed of other than by discharge to a watercourse or must be treated before discharge to a standard acceptable under the Rivers (Prevention of Pollution) Acts, 1951 and 1961 (Anon, 1951, 1961b). Exploratory studies (Pontin & Baxter, 1968)

have shown that there have been considerable variations in the effectiveness of aerobic biological systems in the treatment of piggery wastes and it is considered that part of the problem derives from a lack of information on the biology of the processes and the nature of the wastes, which would permit a rational assessment of the problem.

The importance of microorganisms in purification of wastes was established in the 19th century (Stanbridge, 1954). The principal components of trickling filters and the activated sludge process are microorganisms, nutrients and oxygen, and both processes depend upon a means of keeping these components dispersed yet continuously in contact with each other. In activated sludge contact and aeration is achieved by vigorous agitation, whereas in trickling filters the waste is passed over microorganisms supported on a rigid framework whose interstices provide channels for the exchange and movement of gases as well as drainage of liquid. Bacteria, fungi and protozoa are normally associated with activated sludge; the same groups, together with algae on the top surface, are present in filter beds (Hawkes, 1963). Of these groups the bacteria are predominant in filter beds and are regarded as the basis, both structurally and functionally, of the activated sludge floc (Hawkes, 1963).

Detailed information on the bacteriology of trickling filters is especially scarce because of the difficulty in obtaining representative samples. It is for this reason that the present studies have been concerned with the nature and behaviour of the bacterial populations developing on experimental trickling filters (Gloyna, Comstock & Renn, 1952). The populations

developing as a result of irrigation with domestic and piggery wastes were analysed, and the behaviour of representatives of the major groups of bacteria was investigated to develop a better understanding of their role in the purification of wastes in filter beds.

MATERIALS AND METHODS

GENERAL

Preparation

All chemicals which were used in these studies were obtained from BDH Chemicals Ltd., Poole, Dorset and, unless otherwise stated were of Analar grade. Solutions were prepared in distilled water from a BTL High Capacity all-glass still (Baird & Tatlock (London) Ltd., Chadwell Heath, Essex). pH values were determined with an EIL model 23A direct reading pH meter (Electronic Instruments Ltd., Richmond, Surrey). Glassware was cleaned in Teepol L (Shell Chemicals Ltd.,) followed by thorough rinsing with tap water and then distilled water.

Sterilization

Pipettes were sterilized in an oven at 160° for 3 hours. Other materials, unless otherwise stated, were autoclaved at 121° for 15 min.

Nitrogen determination

Nitrogen was determined by direct Nesslerization of micro-Kjeldahl digests using the method of Minari & Zilversmit (1963). A nitrogen standard solution was prepared by dissolving 707·9 mg of ammonium sulfate in 0·1 N sulfuric acid. Aqueous dilutions of this standard solution were assayed to obtain a standard curve for 5-40 µg nitrogen/sample. The absorbance at 420 mµ was measured in 1 cm glass cuvettes against a reagent blank using a Unicam SP600 spectrophotometer.

Protein determination

Protein was determined by the method of Lowry Rosebrough, Farr & Randall (1951). A standard curve for 5-300 µg protein/ml was obtained by assaying 1 ml samples of aqueous dilutions of bovine serum albumin (Sigma Chemical Company Ltd., London). The absorbance at 750 mµ was measured in 1 cm glass cuvettes against a reagent blank using a Unicam SP600 spectrophotometer.

Carbohydrate determination

Total carbohydrate was determined using the phenol sulfuric acid method (Dubois et al., 1956). A standard curve for 20-80 µg carbohydrate (glucose equivalents)/sample was obtained by assaying 2 ml samples of dilutions of a standard solution of glucose. The absorbance at 490 mµ was measured in 1 cm glass cuvettes against a reagent blank using a Unicam SP600 spectrophotometer.

Chemical Oxygen Demand (COD) determination

The COD was determined by the method described for the examination of industrial wastewaters in Standard Methods for the Examination of Water and Wastewater (Anon, 1965). A 20 ml sample of the waste, or an aliquot diluted to 20 ml was refluxed for 2h with 0·4 g mercuric sulfate, 10 ml 0·25 N potassium dichromate and 30 ml concentrated sulfuric acid. The mixture was then diluted with distilled water to about 140 ml, cooled to room temperature, and the excess dichromate titrated against 0·1N ferrous ammonium sulfate using ferroin indicator (1·485 g 1,10 - phenanthroline monohydrate and 0·695 g

ferrous sulfate heptahydrate dissolved in 100 ml distilled water).

EXPERIMENTAL TRICKLING FILTERS

Apparatus

The experimental trickling filters (Fig. 1 & 2), which were based on the apparatus of Gloyna, Comstock & Renn (1952), consisted of five tubes resting on rollers rotated at 18 rev/min by a Citenco FA 109 shaded pole induction geared motor (Citenco Ltd., Boreham Wood, Herts.). The chassis, which was constructed from Meccano (Meccano Ltd., Liverpool), was adjusted to give the tubes an inclination of 1:25 from the horizontal. The units used were five perspex tubes 61 cm (24 in) long with inside diameters 5·5 cm ($2\frac{1}{4}$ in) (Richard Daleman Ltd., London) the inner surfaces of which were roughened by shot blasting for better adherence of organisms. Wastes were delivered by a Quickfit Instrumentation Peristaltic Pump 10 PP 60 (Quickfit & Quartz Ltd., Stone, Staffs.) into the raised ends of the rotating tubes and the effluent was allowed to drain from the lower ends.

Measurement of Retention

The retention of a soluble tracer was determined in clean perspex tubes. A sodium chloride solution (c.0·3525N) was standardised against 0·0141 N silver nitrate using 5% (w/v) potassium chromate as the indicator. The pump was adjusted to deliver 360 ml/h (approximately 2 gal/day) in 0·2 ml amounts to each tube whilst they were being irrigated with distilled water for 2 h, after a further 30 min 0·2 ml of sodium chloride was substituted for one dose of distilled water and the effluents passing from the lower end of the tube in 0-1, 1-2, 2-3, 3-5 and 5-7 min

were collected separately. Each fraction was then made up to 100 ml with distilled water and titrated against 0·0141 N silver nitrate.

Wastes

The experimental trickling filters were irrigated with settled domestic sewage obtained daily from Bath Corporation Sewage Treatment Works, piggery wastes obtained daily from a local farm, and piggery wastes diluted with tap water. The pH, total nitrogen (Minari & Zilversmit, 1963), total carbohydrate (Dubois et al., 1956) COD (Anon, 1965) and suspended solids (Anon, 1965) were determined for the wastes. The wastes were delivered to each tube at a rate of 2 gal/day which, since each tube had an internal surface area of 1·31 ft^2, was equivalent to 1·52 gal/ft^2/day or 13·68 gal/yd^2/day.

During the period of irrigation, the weight of biological film which was formed in a tube was determined daily by allowing the tube to drain vertically for 25 min and then weighing on a Torbal top pan balance (The Torsion Balance Company (Great Britain) Ltd., Slough, Bucks.). The effectiveness of purification in the trickling filters was monitored daily by determining the COD of the wastes and the effluents.

Sampling the micro flora of the film

The biological film which had developed on the perspex tubes was sampled after the tube had been allowed to drain. The bottom end of the tube was closed with a sterile rubber bung, then 100 ml of $\frac{1}{4}$ - strength Ringer's solution and 50 glass beads were put inside. A second rubber bung was used to close

the other end of the tube which was then shaken vigorously for 5 min to dislodge the film. The suspension of film was poured into a 250 ml conical flask and shaken with glass beads on a wrist action shaker (Baird & Tatlock (London) Ltd.) for 15 min to break up the film.

Isolation of bacteria from the film

The suspension of disrupted film was serially diluted in $\frac{1}{4}$ - strength Ringer's solution and 0·1 ml of each dilution spread with a smooth T-shaped glass rod on the surface of a Petri dish containing 20 ml of solidified nutrient medium. The media used were : Nutrient Agar (Oxoid), Milk Agar (Oxoid), Blood Agar (sterile defibrinated horse blood (Oxoid) was added to liquefied Blood Agar Base (Oxoid) cooled to 45° to give a final concentration of 10% v/v), medium TA (bacteriological peptone (Evans Medical Ltd., Speke, Liverpool) 2·5 g; yeast extract (Oxoid L21) 2·5 g; agar (Oxoid No. 3) 15·0 g; distilled water 1 l; pH 7·2) and medium TAG (an aqueous solution of glucose was sterilsed by Seitz filtration and added to medium TA (liquefied and cooled to 45°) to give a final concentration of 0·1 % w/v). Six 0·1 ml volumes of each dilution were plated on each medium, three plates were incubated at 25° and the others at 37°, and colonies were counted at intervals up to 10 days.

Isolates for identification were taken from plates of medium TA which had counts of 30-120 by the sixth day of incubation at 25°. A random sample was obtained by dividing each plate into four sectors and subculturing every colony from one quadrant of each plate (Baird-Parker, 1962) on to plates of medium TA. The cultures were purified by successive

transfer in 0·25% (w/v) peptone water and streaking on medium TA.

Diagnostic tests used in the identification of bacteria from the film

A 48h slope culture on medium TA was used as an inoculum in all experiments and all tests were incubated at 25°.

The Hucker modification of the Gram-staining procedure (Committee on Bacteriological Technic, 1957) was used for cytological observations. When a stained film did not give a suitable preparation for determination of the shape and arrangement of an organism isolates were negatively stained by the method described by Cruickshank (1965). Capsulation was demonstrated by negative staining (Duguid,1951) with Pelikan indian ink (Wagner Günther AG Pelikan-Werk, Zurich, Switzerland), counterstained for 30 sec with Loeffler's Methylene Blue. Reserve materials were stained with Sudan Black B (Burdon, 1946) and flagella by the method of Rhodes (1958).

Motility was determined by examining, by phase contrast microscopy, a hanging drop preparation of an 18 h peptone (0·25 % w/v) water culture.

Kovac's (1956) method was used for the oxidase test. A platinum loopful of cells was rubbed on filter paper moistened with a 1% (w/v) aqueous solution of p-amino- N:N - dimethyl aniline. The development of a blue colour within 30 sec was recorded as positive.

Carbohydrate breakdown by Gram-negative organisms was tested in the medium of Hugh & Leifson (1953) and for Gram-positive organisms in the Baird-Parker (1963) modification of this medium.

The substrate, glucose, was sterilized by Seitz filtration and added to the liquefied medium (cooled to 45°) to give a final concentration of 1% (w/v)

Anaerobic hydrolysis of arginine was indicated by production of alkalinity in Thornley's (1960) medium.

Maintenance of cultures

Bacterial cultures were kept under sterile liquid paraffin on slopes of medium TA in a refrigerator at 5°, and were transferred to fresh slopes at intervals of 6 months.

STUDIES WITH REPRESENTATIVES OF THE MAJOR GROUPS OF BACTERIA

Media

All experiments were done in media containing a mineral base which consisted of phosphate buffer (0·5 M, pH 6·8) 80 ml; Hutner's base (Cohen-Bazire, Sistrom & Stanier, 1957) 20 ml; ammonium sulfate 1·0 g; distilled water 1 l. This mineral base differed from that used by Stanier, Palleroni & Doudoroff (1966) and Baumann, Doudoroff & Stanier (1968 a, b) only in the use of 0·5 M phosphate buffer. Compounds which were tested for their ability to support growth were added to give a final concentration of 0·1 % (w/v).

The principal medium used in these investigations was a synthetic sewage medium similar to that of Butterfield, Ruchhoft & McNamee (1937). It contained bacteriological peptone (Evans) 0·3 g; yeast extract (Oxoid) 0·2 g; urea 0·05 g; mineral base 1 l.

Solid media were of the same composition as liquid media but incorporated 1·5% (w/v) agar (Oxoid No. 3)

Additional diagnostic tests used in the identification of representatives of the major groups of bacteria

A 48 h slope culture on medium TA was used as an inoculum in all experiments and all tests were incubated at 25°.

Compounds which were tested for their ability to support growth were incorporated in mineral base agar to give a final concentration of 0·1 % (w/v), and a strain was considered to be capable of growth on a compound when growth occurred through

three successive transfers on that medium.

Pigment production in pseudomonads was detected by streaking the organism on slopes of medium A and medium B of King, Ward & Raney (1954). Medium A favour the production of phenazine (a blue water-insoluble pigment) whilst medium B suppresses the production of phenazine and encourages the production of fluorescent pigments.

Galatin hydrolysis was tested by inoculating the organism in the centre of the surface of a plate of modified Frazier's gelatin agar (Smith, Gordon & Clark, 1952) and incubating for 14 days. The plate was then flooded with acid mercuric chloride solution (Frazier, 1926), a clear area around the inoculum indicated hydrolysis of the gelatin.

Spreading growth was tested by inoculating the centre of the surface of a well dried plate of a medium containing yeast extract 1·0 g; agar (Oxoid No. 1) 10 g; distilled water 1 l; pH 7·2. On this medium typical spreading growth takes the form of a thin film extending over the surface from the site of inoculation (Hendrie, Mitchell & Shewan, 1968).

Inocula

Cultures for use as inocula were grown overnight in flasks of synthetic sewage medium. From these cultures an inoculum equivalent to 10 µg of nitrogen was transferred to each flask required in the experiment. When two organisms were to be grown together an inoculum equivalent to 5 µg of nitrogen from each organism was used.

Incubation

The temperature of incubation was 25° in all experiments, liquid media were dispensed in 25 ml amounts in 250 ml Erlenmeyer flasks capped with Gunn sidegrip closures (C.E. Payne & Sons Ltd., Clapham, London). Aerated cultures were incubated in a Gallenkamp orbital incubator (A. Gallenkamp & Co. Ltd., Christopher Street, London) at 250 rev/min with a diameter of orbit of 32 mm.

Estimation of numbers of viable bacteria

Viable counts were done by the method of Miles & Misra (1938) using the hypodermic needle type of dropping pipette (Astell Laboratory Services Ltd., Catford, London) as described by Davis & Bell (1959).

In most experiments, however, the amount of nitrogen was used as the index of bacterial growth. This was calculated from the absorbance of a culture by reference to standard curves of total nitrogen (Minari & Zilversmit, 1963) against absorbance at 610 mμ (measured in 1 cm glass cuvettes against a water blank using a Unicam SP 600 spectrophotometer) which had been prepared for each organism.

Preparation of culture filtrates for analysis

Reduction of COD, protein and carbohydrate levels as a result of bacterial activity in synthetic sewage medium were determined at intervals after inoculation. To arrest biological activity 1 ml of 1% (w/v) mercuric chloride was added to each flask, and then 20 ml of the culture was filtered through a membrane (pore size 0·45 μ, Oxoid). The membrane was

then rinsed twice with 5 ml amounts of distilled water and the culture filtrate made up to 40 ml before analysis.

RESULTS

STUDIES WITH EXPERIMENTAL TRICKLING FILTERS

Attempts were made to purify the wastes of pigs by passage over the rotating tubes of an apparatus (Gloyna, Comstock & Renn, 1952) which is considered to simulate the conditions obtaining in a biological filter bed (Tomlinson & Snaddon, 1966). Trickling filters are widely used in the treatment of domestic sewage but there is little information concerning their application to farm wastes, particularly those derived from intensively housed pigs. Therefore, in addition to piggery wastes, the rotating tubes were irrigated with domestic sewage to allow comparisons to be made with results from previous investigations of trickling filters.

Data concerning the strength and composition of the piggery and domestic wastes used in this study and piggery wastes which have been investigated by other workers are presented in Table 1. These analyses show that piggery wastes are not only far stronger than domestic sewage but vary greatly amongst themselves as to their composition. The inherent variations in the factors that affect wastes (feed composition, environment, etc.) and in the different methods of waste handling make it impossible to define adequately a typical piggery waste. However it must be recognised that an understanding of the properties of piggery wastes is essential to the development of methods for their handling and disposal, and that procedures used in the treatment of domestic sewage cannot be applied without modification to piggery wastes.

Consideration of the experimental and practical trickling filters

Since the object of this investigation was to obtain a better understanding of trickling filters it was considered desirable to choose a rate of treatment corresponding to that at which wastes might be applied to a filter bed in practice. When the rotating tubes apparatus was used for the purification of domestic sewage by Gloyna et al. (1952), the volume (hydraulic loading) of waste applied to each tube was 7 l/day. Purification, measured as the reduction in Biochemical Oxygen Demand (BOD) of the waste, was similar in terms of equal wetted areas to a 6 ft deep bed of 2 in stones irrigated at the same hydraulic loading (192 ml/ft^2/h). At a lower hydraulic loading of around 5 l/day (137 ml/ft^2/h), Tomlinson & Snaddon (1966) found that the rotating tubes had a performance, in terms of removal of BOD and suspended solids, equivalent to a 6 ft deep bed of 1 in stones irrigated at 15·9 ml/ft^2/h (60-100 gal/yd^3/day). In all experiments in the present study the tubes were irrigated at a hydraulic loading of around 8 l/day, higher than that used by Gloyna et al. (1952) or Tomlinson & Snaddon (1966), because it was hoped to simulate the high hydraulic loadings applied to plastic-packed filter towers.

It was also considered that it might be of value in the interpretation of the observed behaviour of the experimental filters to know the time for which an added tracer was retained in it. For this reason the retention of a soluble tracer (Na CL) was estimated in clean tubes irrigated at 8l/day. The results of the recovery of the tracer are presented in Table 2.Examination of retention characteristics

(Eden, Brendish & Harvey, 1966) has shown that the data almost invariably follow a log-normal distribution. This relation can be transformed into a straight line by plotting cumulative recovery on a probability scale against time on a logarithmic scale. The data from Table 2 was plotted in this manner and a straight line fitted by the method of least squares. The product moment correlation coefficient (r) was calculated as 0·99 and the time required to recover 50% of the added tracer was 3·4 min (Fig.4).

This retention period compared reasonably well with those of Eden, Truesdale & Mann (1966) in which 50% of tracer (bromide - Br 82) was recovered from a 7 ft tower in 5 min packed with a plastic medium ("Surfpac", Hydronyl Ltd., Stoke-on-Trent, Staffs). The performance of the rotating tubes apparatus irrigated with domestic sewage at 8 l/day (287 ml/ft^2/h) in terms of COD removal, was also equivalent to that in the plastic-packed tower irrigated at 1,000 - 1,500 gal/yd^3/day (273-410 ml/ft^2/h).

Film build-up as a result of irrigation with domestic sewage

While the rotating tubes were irrigated with settled domestic sewage (Table 1) a slimy film formed on their inner surfaces. Accretion of film began at the end of the tube nearest the point of introduction of the waste and extended progressively downwards until the whole innner surface was covered by the third or fourth day of irrigation. The amount of film present on the Perspex tubes was determined daily by weighing the tube after it had been left in the vertical position for 25 min, by which time (Fig.3) the greater part of the surface water, other than that held by capillarity etc.,

had drained off. After a lag phase of around 24 h from the beginning of irrigation the weight of film in the tube increased exponentially with time (Fig. 5). Throughout investigations of periods of irrigation of up to five weeks, the weight of film continued to increase slowly and a constant weight over a period of time was never achieved (Fig. 6, Appendix 1). In conventional stone-packed filter beds, grazing by protozoa is considered to play a major part in controlling the amount of film (Hawkes, 1963). However, in this study, examination of films, either stained preparations or by phase contrast microscopy, did not reveal any protozoa. Although large amounts of film broke away occasionally (Fig. 6) it is probable that the amount of film on the rotating tubes was controlled principally by small amounts continually becoming detached and carried out with the effluent.

The film, which was initially opaque but developed a dark brown colour within 48 h of irrigation, was always moist, even after drainage, and slimy to the touch. The morphology and arrangement of the organisms in the film was studied by phase contrast microscopy and as heat fixed preparations which had been stained by Gram's method or with dilute Carbol Fuchsin or Loeffler's Methylene Blue. Unfortunately, because it was necessary to smear the film on the slide for microscopic examination, preparations were never as clear as those which have been obtained for activated sludge (Adamse, 1966) and it was impossible to discern whether there was any organisation of the organisms (e.g. microcolonies) within the film. The predominant organisms were Gram-negative cocoid rod and rod-shaped

bacteria embedded in capsular material. Occasionally yeasts and fungal mycelium were seen but in some preliminary experiments it was not found possible to isolate any moulds by plating on Potato Dextrose Agar (Oxoid) or on modified Czapek Dox Agar (Oxoid). The coryneform organisms which were isolated from the film (see page 25) and identified principally from their characteristic morphology were not seen in microscopic examinations. However, in two investigations of activated sludge where coryneforms were considered to be major contributors to the microflora (Van Gils, 1964; Adamse, 1966) it is not recorded whether coryneforms were detected by microscopy. In the earlier of these studies (Van Gils, 1964) large spherical or lemon-shaped cells were seen in the activated sludge, and it may be that these were giant cells (cystites) of Arthrobacter (Mulder & Athenuisse, 1963).

In order to identify and enumerate the different organisms of the film it was considered desirable to select bacteria from the conditions which promoted the highest recovery of viable organisms. One of the principal difficulties in studying the microflora of films or of activated sludge is to disrupt the film and release viable organisms from the capsular material. In this study the film from a tube was stripped off and disrupted by shaking with glass beads for periods of up to 20 min. The effect of disruption in the recovery of viable organisms was tested on Nutrient Agar and medium TA incubated at 25° and 37° (Fig. 7). It was found that disruption had no effect on the number of viable organisms

recovered at 37°, which would suggest that organisms developing at this temperature are not associated with the indigenous organisms which are embedded in the capsular material. Recovery at 37° neither differed amongst the five media tested (medium TA, medium TAG, Nutrient Agar, Blood Agar and Milk Agar), nor increased as a result of incubation beyond 2 days (Table 3). However the numbers of viable organisms recovered by incubation at 25° were always higher than those recovered at 37°, and in some media increased as a result of incubation for up to 6 days (Fig. 8). In fact, the medium with the lowest concentration of nutrients, medium TA, resulted in the highest recovery of viable organisms at 25°. Since these counts were not effected by addition of glucose (medium TAG) it would suggest that obligatory saccharolytic organisms were uncommon in the film. It was concluded that the conditions which promoted the highest recovery of viable organisms indigenous to the film involved shaking the film with glass beads for 15 min for disruption (Fig.7) plating out in medium TA and incubating at 25° for 6 days (Fig. 8). The majority of the organisms isolated by this means were morphologically (Gram-negative to variable coccoid rod and rod-shaped bacteria) very similar to those seen by microscopic examination of the film.

Details of the tests which were of diagnostic value in the identification at generic level of organisms recovered from the experimental trickling filters are presented in Table 4 and the data obtained from these tests were used to devise the determinative key set out in Table 5. The results of the identification of 400 isolates from films which had developed on the experimental trickling

filters irrigated with domestic sewage are presented in Fig.9 Of the isolates which were identified 200 had been sampled from a young film (sampled 2 days after beginning irrigation) and the rest from a mature (see page 106) film (sampled 21 days after beginning irrigation). It was considered that the proportion of organisms which were capable of rapid breakdown of the waste would increase as the film matured and that those whose proportion dwindled would be unsuccessful competitors in the ecosystem. The organisms which increased in numbers were either aerobic Gram-negative to variable, non-pigmented, non-motile coccoid rods occurring mainly in pairs, which were identified with Acinetobacter; or of a group of aerobic Gram-negative, yellow pigmented, non-motile rods to which none of the generic names defined in Bergey's Manual of Determinative Bacteriology (Breed, Murray & Smith, 1957) were wholly applicable. All other groups decreased in their numerical importance except the coryneforms (identified principally by their irregular club or wedge-shaped cells and irregular staining) which were present in the same proportion in young and mature films. Whereas it seems likely that Acinetobacter and the yellow-pigmented Gram-negative rods comprise an association which is responsible for waste purification in trickling filters the contribution made by the coryneforms is uncertain. Certainly they do not conform to the general shape and arrangement of the organisms detected by microscopic examination of the film. However, it is an important consideration that in other investigations (Van Gils, 1964, Adamse, 1966)coryneforms have been shown to be primarily responsible for waste purification in the activated sludge process yet have not been detected by microscopic

examination of the flocs.

Film build-up as a result of irrigation with piggery wastes

A dark brown film covering the whole inner surface of the experimental trickling filters developed as a result of irrigation with domestic sewage. However, when they were irrigated with piggery waste (Table 1) or piggery waste diluted to 2,000 mg/l COD films which developed were grey and patchy. Over the first 48 h of irrigation the weight of film increased in the same manner as the film developing as a result of irrigation with domestic sewage, but it then levelled off at around 10 g (Fig. 5).

Because of this scant film developed as a result of irrigation with normal or diluted piggery waste it was decided to develop a film by irrigation with domestic sewage and then acclimatize this film to piggery wastes. Tubes were irrigated with domestic sewage for 21 days and then a piggery waste diluted to 280 mg/l COD was applied to the films. Over the following weeks (Appendix 2) the concentration of the piggery waste was gradually increased to 2,800 mg/l COD. However, as the strength of the piggery waste was increased the dark brown colouration, characteristic of domestic sewage films, changed to the same grey colour as the scattered patches of film which had developed when clean tubes were first irrigated with piggery wastes. These findings support the information presented by Heukelekian & Crosby (1956) who found that films grown in high nutrient concentrations were watery and tended to become detached more easily than those grown in low nutrient concentrations.

Over the first 14 days the strength of the diluted piggery waste (Appendix 2) was increased from 280 mg/l COD to 700 mg/l COD, and at this stage the film was sacrificed for the identification and enumeration of its microflora. The methods used for the isolation and identification of microorganisms from this film were the same as those used for films developing as a result of irrigation with domestic sewage. The results of the identification of 95 isolates recovered from this film are presented in Fig. 10. They show that under these conditions one group of organisms, identified with Acinetobacter dominated the flora. The yellow-pigmented rods, which were the other principal group associated with films irrigated with domestic sewage were no longer of numerical importance after the film had been acclimatized to dilute piggery waste (Fig.10). It is tempting to suggest that the decrease in numerical importance of these yellow-pigmented organisms is responsible for the loss of colouration when films are irrigated with piggery wastes especially since Adamse (1966) attributes the brown colouration of activated sludge to a group of Gram-negative yellow-pigmented rods which were identified with Flavobacterium. It is also possible, since purification of piggery waste is lower than that of domestic sewage of the same strength (Fig.11), that the yellow-pigmented rods play a major role in the purification of wastes and that in their absence Acinetobacter is much less effective.

Purification of wastes in experimental trickling filters

During periods of irrigation with wastes of either domestic or piggery origin the COD of the wastes and the effluents were monitored daily, and the effectiveness of

purification in the experimental trickling filters recorded as the percentage by which the COD of the waste had been reduced.

It was found that the effectiveness of purification of domestic sewage remained at about the same level (c. 60%) as long as there was 20 g or more of film in the tube (Fig.12). Less than 20 g of film only occurred during the initial periods of irrigation (Fig. 5, 6, Appendix 1) before the whole innner surfaces of the tubes had been clothed in film. Therefore it can be concluded that the degree of purification of domestic sewage in the experimental trickling filters is dependent upon the surface area rather than the weight of mature film over which the waste passes.

A further variable was the strength of the domestic sewage, usually it had a COD between 400-700 mg/l but occasionally it was as low as 240mg/l and as high as 830mg/l. It was found that the effectiveness of purification increased for stronger domestic wastes (Fig.11) within these limits.

STUDIES WITH REPRESENTATIVES OF THE MAJOR GROUPS OF BACTERIA

Organisms

Three organisms isolated from the films developed on the experimental trickling filters were chosen for further study. Two were selected to represent the groups of organisms which were of numerical importance in the films and which were considered to be effective in the purification of wastes, Acinetobacter and the yellow-pigmented Gram-negative rods. The third organisms was chosen to represent Pseudomonas because these organisms have frequently been considered of importance in aerobic waste treatment processes (McKinney & Weichlein, 1953; James, 1964).

The properties used in the identification of these organisms are summarized in Table 6. The representative of Acinetobacter (hereafter referred to as Acinetobacter 4) was identified with Phenon 4 of Thornley (1967), a group which is considered (Thornley, 1967) to include isolates from a wide variety of protein foods. The pseudomonad was identified with Pseudomonas putida (Stanier, Palleroni & Doudoroff, 1966), a species which has most frequently been isolated from soil by enrichment with a wide variety of compounds. However due to the inadequate nature of normal bacteriological methods for differentiating yellow-pigmented Gram-negative rods (Hendrie, Mitchell & Shewan, 1968) it was not possible to identify the isolate used in this study (hereafter referred to as the yellow rod) with any of the well defined genera listed in Bergey's Manual of Determinative Bacteriology (Breed, Murray & Smith, 1957) or those discussed

by Hendrie, Mitchell & Shewan (1968).

Competition between Acinetobacter 4 and Pseudomonas putida in synthetic sewage

In the first of these experiments the growth of pure cultures of Ac. 4 and Ps. putida was investigated in 250 ml conical flasks containing 25 ml synthetic sewage medium. Growth was studied in standing flasks which were incubated at 25° and in flasks which were vigorously shaken on an orbital shaker (250 rev/min, diameter of orbit 32 mm) at 25°. In standing flasks of synthetic sewage Ac.4 in pure culture grew much less rapidly (Table 7) and exhibited a longer lag phase than pure cultures of Ps. putida (Fig.13). However, when the two organisms were incubated under conditions of brisk aeration their growth rates differed only very slightly (Table 7, Fig. 14).Aeration had little effect on the growth curves of Ps. putida, but resulted in a considerable increase in the growth rate of Ac.4.

This investigation was continued by inoculating flasks of synthetic sewage with equal amounts (in terms of Kjeldahl nitrogen) of both organisms. Growth was monitored by viable counts (Miles & Misra, 1938) on two media, medium TA on which both organisms developed, and a medium containing 0·1%(w/v) glucose as the sole carbon and energy source (medium GMBA) on which only the pseudomonad was capable of growth. By this means the numbers of pseudomonads in the mixed population could be determined. In standing flasks of synthetic sewage viable counts on the two recovery media were the same (Fig.15) which, confirmed by results from microscopic examination of colonies selected from the plates, suggests that Ps. putida made up the bulk of the population.

This can be explained if Ps. putida had a faster growth rate in synthetic sewage than Ac.4, a view which was corroborated by the evidence obtained from pure culture studies (Fig. 13). Rapid growth of the pseudomonads, which are motile and rise to the surface as a result of aerotactic response (Baumann, 1968), diminishes the oxygen supply for non-motile organisms such as Ac.4 and arrests their development. In aerated flasks of synthetic sewage the viable counts in medium GMBA (Glucose Mineral Base Agar) remained low whilst counts on medium TA increased (Fig. 16). This suggests that under conditions of aeration Ac.4 became the dominant organisms in a mixed population of Ac.4 and Ps. putida. If Ac.4 had a faster growth rate under aeration then Ps. putida, or was present in greater numbers initially, this could be explained easily. However in the experiments a standard equal inoculum of each organism was employed, and the growth rate of Ac.4 and Ps. putida in pure cultures had been shown to be much the same (Table 7). The only difference exhibited in pure culture (Fig. 14) was that Ac. 4 had a marginally shorter lag phase than Ps. putida, but it is felt that this is unlikely to provide a complete explanation for the dominance of Ac. 4 under conditions of aeration.

Growth of mixed cultures of the yellow rod and Acinetobacter 4 and Ps. Putida

In these experiments flasks of synthetic sewage were inoculated with equal amounts (in terms of Kjeldahl nitrogen) of the yellow rod and Ac. 4 or Ps. putida and incubated at 25° on an orbital shaker. Since its pigmentation distinguished

the yellow rod from Ac. 4 or Ps. putida (Table 6), viable counts on medium TA were adequate for monitoring the growth of organisms in mixed populations. However, although the inocula represented the same amount of nitrogen of each organism, the smaller cell size of the non-pigmented organisms resulted in their contribution to the inocula being larger than that of the yellow rod.

The lag phases before the populations of Ac.4 or Ps. putida began to increase were of much the same duration as that of the yellow rod. Ac. 4 and the yellow rod grew in mixed culture at about the same rate (Fig. 17) and the numbers of yellow rods at no time equalled or exceeded the numbers of Ac. 4 (Fig. 17), but Ac. 4 failed to achieve as high a stationary phase population as it had in pure culture (Fig. 14). However Ps. putida did not grow well in mixed cultures with the yellow rod. The yellow rod had a higher growth rate (Fig. 18) than the pseudomonad and attained a larger population in the stationary phase. The growth rate of Ps. putida in mixed cultures with the yellow rod (Fig. 18) was somewhat lower than that of Ps. putida in pure culture (Fig.14). These results would suggest that Ac. 4 is more likely than Ps. putida to be capable of living in close association with the yellow rod and may help to explain why pseudomonads were not present in this study of bacterial flora of film which developed in experimental trickling filters (Fig. 9, 10).

Removal of COD, protein and carbohydrate from synthetic sewage by Acinetobacter 4 and Pseudomonas putida

Flasks of synthetic sewage were inoculated with either Ac. 4 or Ps. putida and incubated at 25° on an orbital shaker. At intervals after inoculation flasks were removed from the shaker and the organisms separated from the culture filtrate by membrane (pore size 0·45 μ) filtration. The culture filtrate was then made up to 40 ml with distilled water and analyzed for COD, protein and carbohydrate. Reduction of COD, protein and carbohydrate levels occurred when the organisms were dividing but apparently not in the lag or stationary phases of growth (Appendix 5). The removal of these constituents from the synthetic sewage has been expressed as the percentage by which they were reduced as a result of bacterial activity, and this has been plotted against the amount of Kjeldahl nitrogen represented by each organism. (Fig. 19, 20, 21) Removal of COD (Fig. 19) and protein (Fig. 20) was much the same by both Ac. 4 and Ps. putida, but substantially less carbohydrate was removed by Ac.4 than by the pseudomonad (Fig. 21).

However, in achieving these levels of substrate removal the population of Ac. 4 in the stationary phase of growth was equivalent to only about half the amount of nitrogen attained by Ps. putida in the stationary phase. In trickling filters there may be a tendency towards an excess accumulation of film which results in occlusion of the void spaces, and a decline in the efficiency of purification of the waste. The conditions under which trickling filters operate most efficiently should therefore be selective for

organisms which are capable of rapid utilization of the organic matter in the waste without too great a level of film build-up. Therefore the removal of substrates from synthetic sewage per unit of bacteria must be regarded as an important consideration. A feature of bacterial growth, which was established by Monod (1942) in media containing a single carbon substrate, is that the growth rate is a constant fraction of the rate of utilization of the substrate. This can be expressed as

$$dx/dt = - Y\, ds/dt,$$

Where x is the concentration of bacteria, s is the concentration of substrate, t is time and Y is called the yield constant. Over any finite period of growth

$$\frac{\text{weight of bacteria formed}}{\text{weight of substrate used}} = Y.$$

Although synthetic sewage is a complex medium, the yield constant can be of use in expressing the efficiency with which organisms remove its organic constituents. The removal of two gross categories of substrates, proteins and carbohydrates, from synthetic sewage, and the reduction of its COD, an over-all parameter of its pollution capability, were monitored as the bacterial populations increased. An organism with a low yield constant as a result of fewer cells being formed per unit of substrate used would have a smaller tendency towards excess film accumulation in a filter bed. In terms of removal of protein from synthetic sewage Ac.4 had a lower yield constant (i.e. more efficient) than Ps. putida; but the yield constant of the former was higher(i.e. less efficient) in terms of removal of carbohydrates. However since there was much less carbohydrate than protein in synthetic

sewage, Ac. 4 had the lower yield constant in terms of the parameter of over-all pollution capability, COD, and was therefore the more efficient of the two organisms in the purification of the waste. It is tempting to suggest that in a carbohydrate-rich waste Pseudomonas might be the dominant organism because of its lower yield constant in terms of carbohydrate utilization. It has been suggested (Adamse, 1966) that proteinaceous wastes are dominated by organisms which resemble Acinetobacter whilst carbohydrate wastes favour Pseudomonas as well.

Removal of COD, protein and carbohydrate from culture filtrates by pure cultures of Acinetobacter 4 and Pseudomonas putida

The reduction of the COD of synthetic sewage as a result of the activity of pure cultures of Ac. 4 and Ps. putida (Fig. 19) was never as high as that achieved for domestic sewage by the populations in the experimental trickling filters (Fig. 11). Therefore it was decided to investigate whether further purification could be effected by Ac. 4 and/or Ps. putida from culture filtrates of synthetic sewage in which they had already grown.

The organisms were inoculated into flasks of synthetic sewage and incubated at 25° on an orbital shaker until their maximum populations were reached. The culture filtrates were separated from the organisms by membrane filtration and dispensed (in 25 ml amounts) into sterile 250 ml flasks. Each organism was then inoculated into its own culture filtrate and into the culture filtrate derived from the other organism. The flasks were incubated at 25°

on the orbital shaker and removal of COD, protein and carbohydrate monitored.

Both Ac. 4 and Ps.putida grew in their own and each others' culture filtrates and attained similar levels of cell nitrogen as they had in the original medium (Appendix 6). Removal of COD (Fig. 22) and protein (Fig. 23) by both organisms was much the same as in the original synthetic sewage, yield constants for Ac. 4 were the lower of the two. This would suggest that cessation of growth in synthetic sewage did not result from exhaustion of the nutrients. Ps. putida also effected a further carbohydrate reduction (Fig. 24) in both culture filtrates, but any carbohydrate remaining in either culture filtrate after the first growth cycle must have been unavailable to Ac. 4 because no further removal was effected (Fig. 24).

Many other investigators (Penfold, 1914; Graham-Smith 1921) have recorded that organisms can be separated from fully grown cultures and the filtrate used to support further growth on reinoculation. In general the decrease in growth rate at the end of the logarithmic phase and the final cessation of all growth results either from exhaustion of nutrients or from accumulation of toxic products (Dean & Hinshelwood, 1966). However in this study nutrients were not exhausted at the end of the first log phase of growth and no special treatment was required to neutralize toxic products. To explain this type of situation it has been suggested in Topley and Wilsons' Principles of Bacteriology and Immunity (Wilson & Miles, 1946) that, in cultures incubated aerobically,

growth continues until the increasing concentration of organisms renders it impossible for each individual organism to obtain sufficient oxygen to meet its requirements.

FIGURES

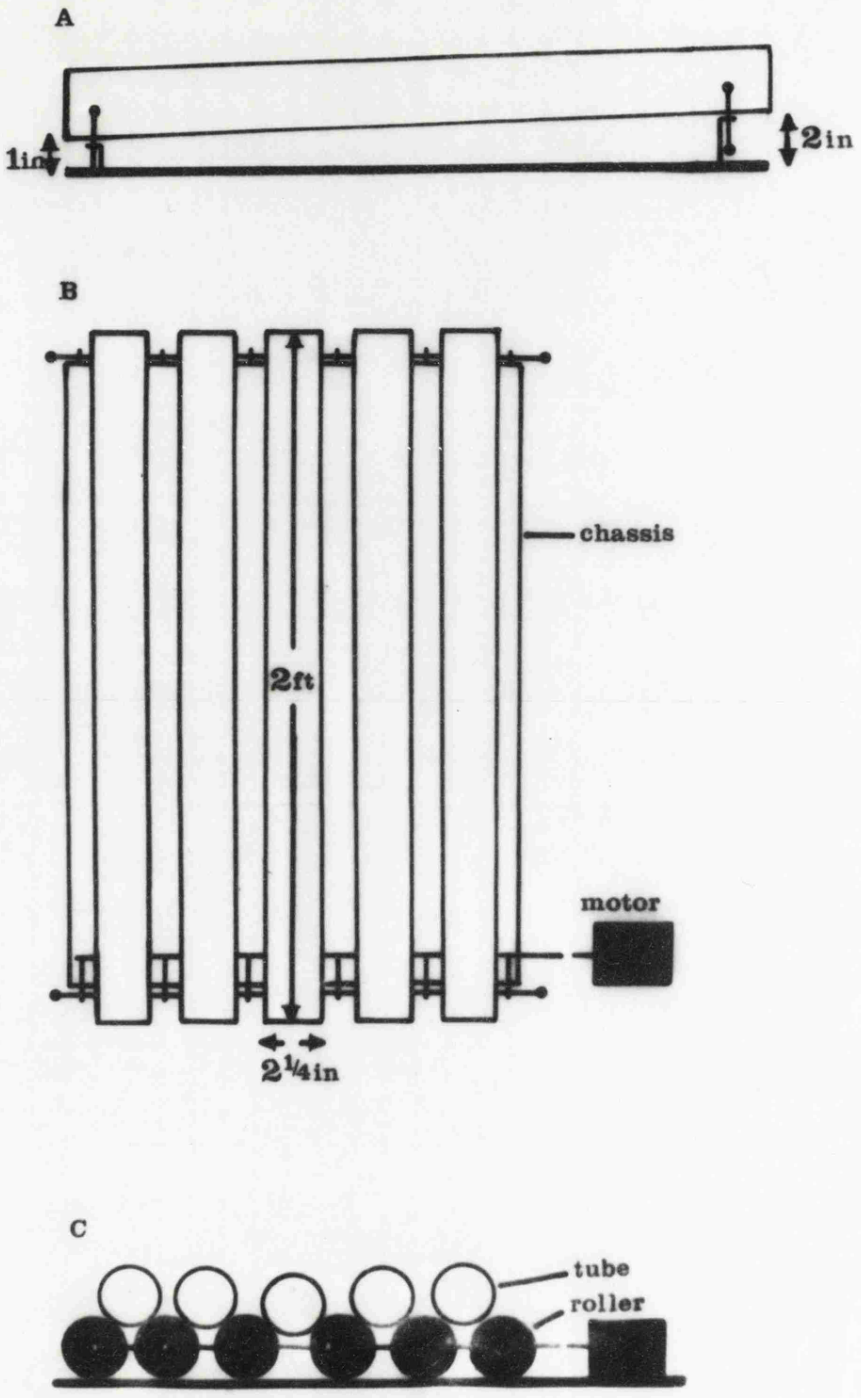

Fig. 1. Schematic plan of experimental trickling filters. A, side view; B, top view; C, front view.

Fig. 2. Experimental trickling filters.

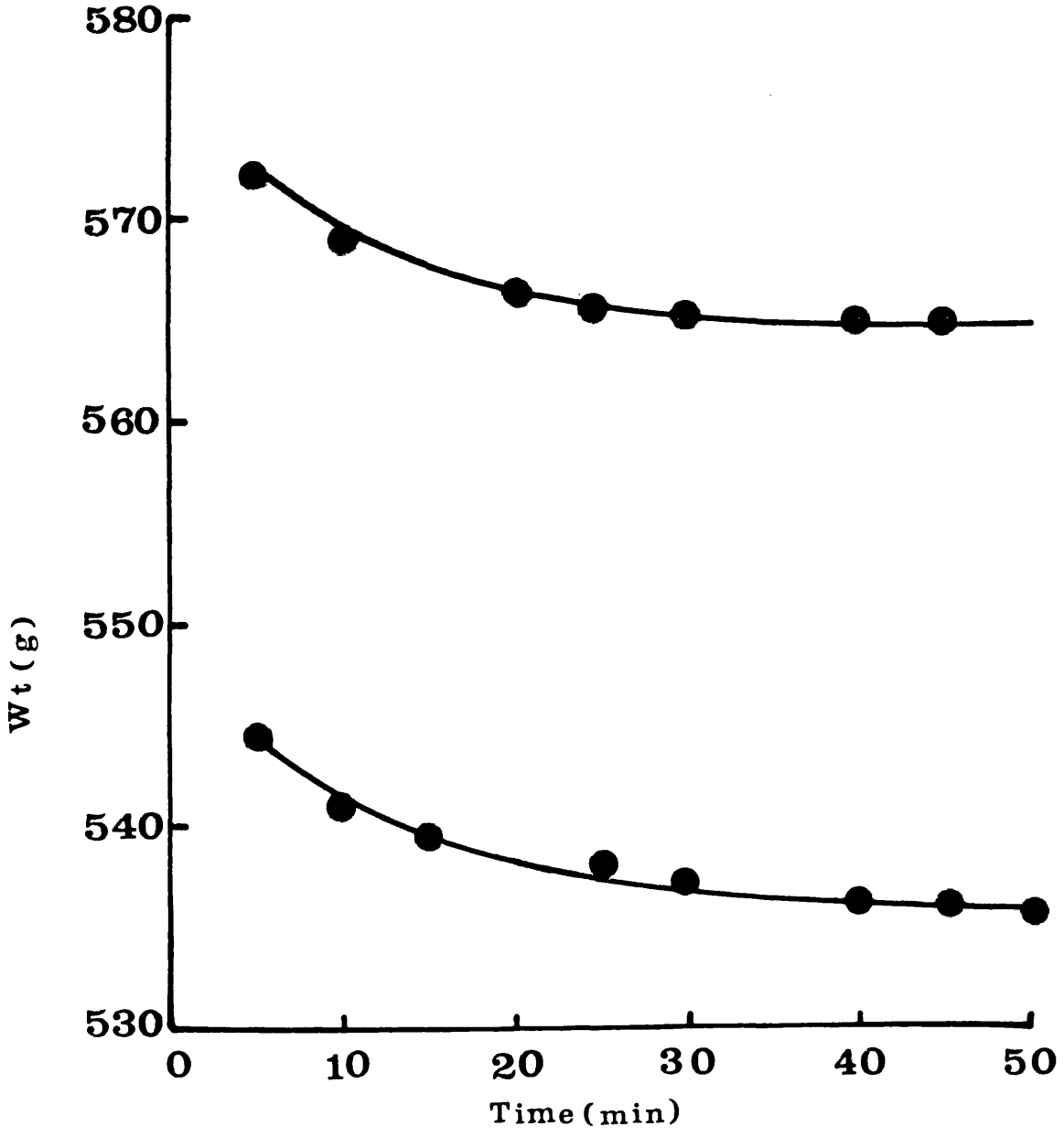

Fig. 3. The effect of the period of drainage on the loss of surface water from Perspex tubes clothed with microbial films.

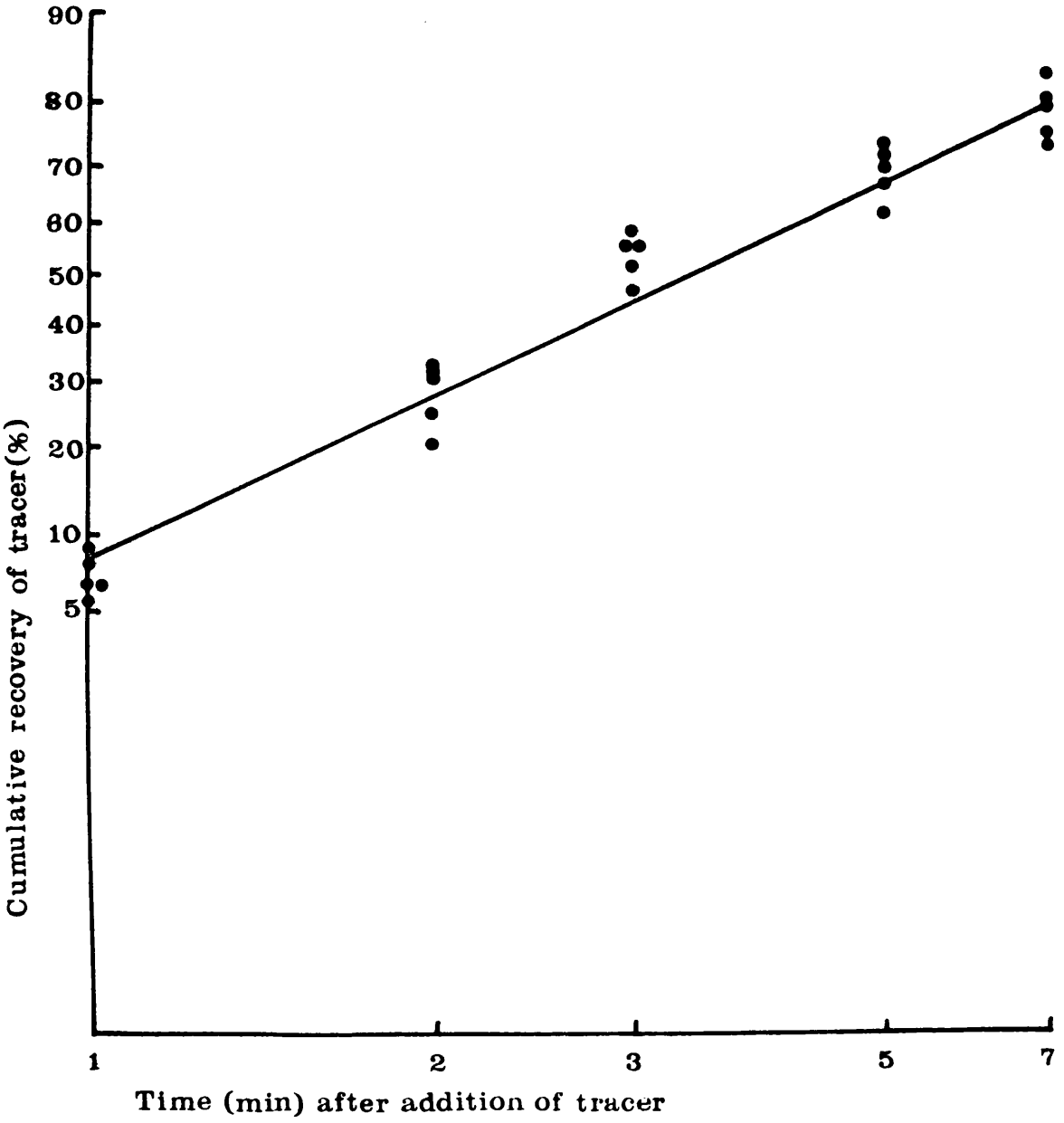

Fig. 4. The retention of a tracer (Na Cl) in the experimental trickling filters.

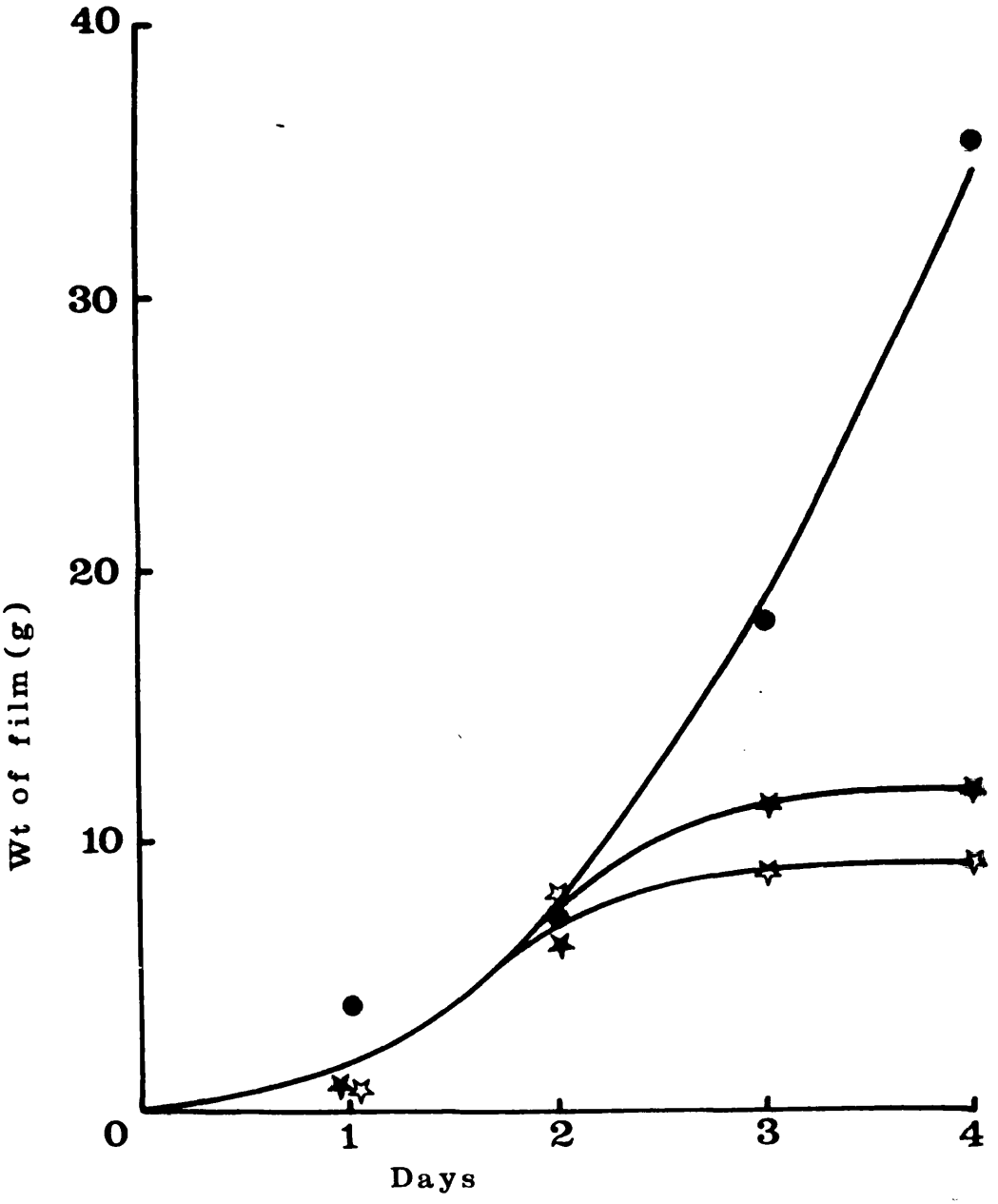

Fig. 5. Initial accretion of films on the Perspex tubes. Closed circles, irrigated with domestic sewage; open stars, irrigated with piggery waste (8,800 mg/l COD); closed stars, irrigated with dilute piggery waste (2,000 mg/l COD).

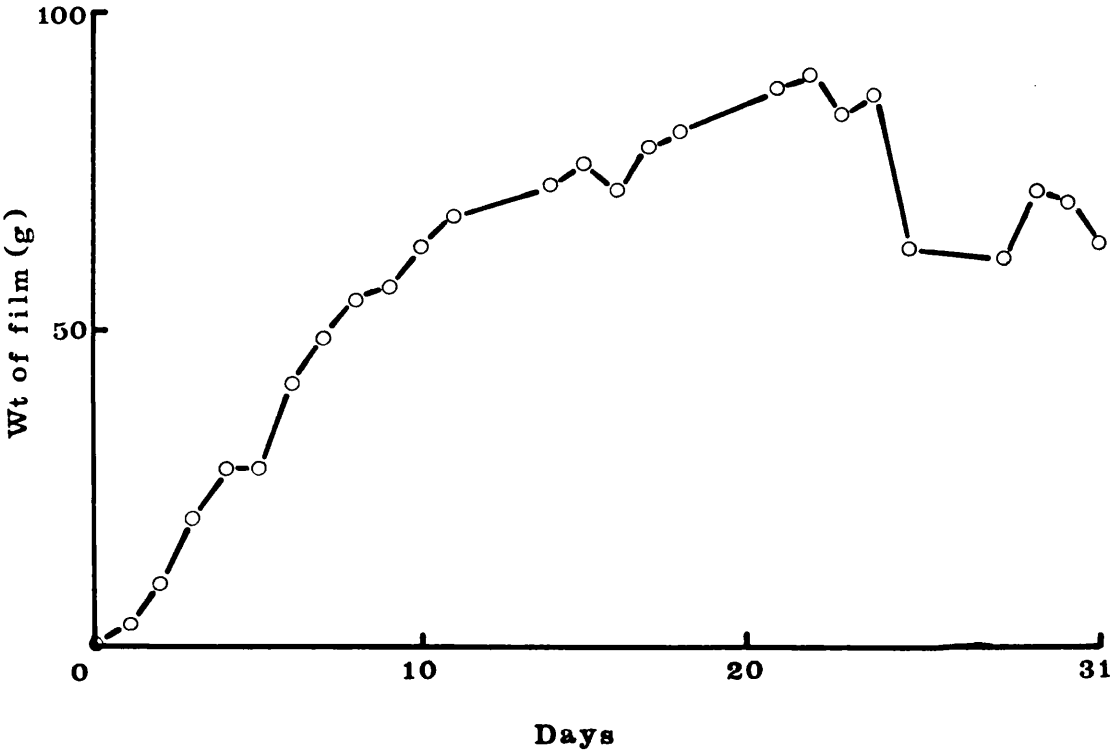

Fig. 6. Accumulation of film on a Perspex tube experimental trickling filter irrigated with domestic sewage.

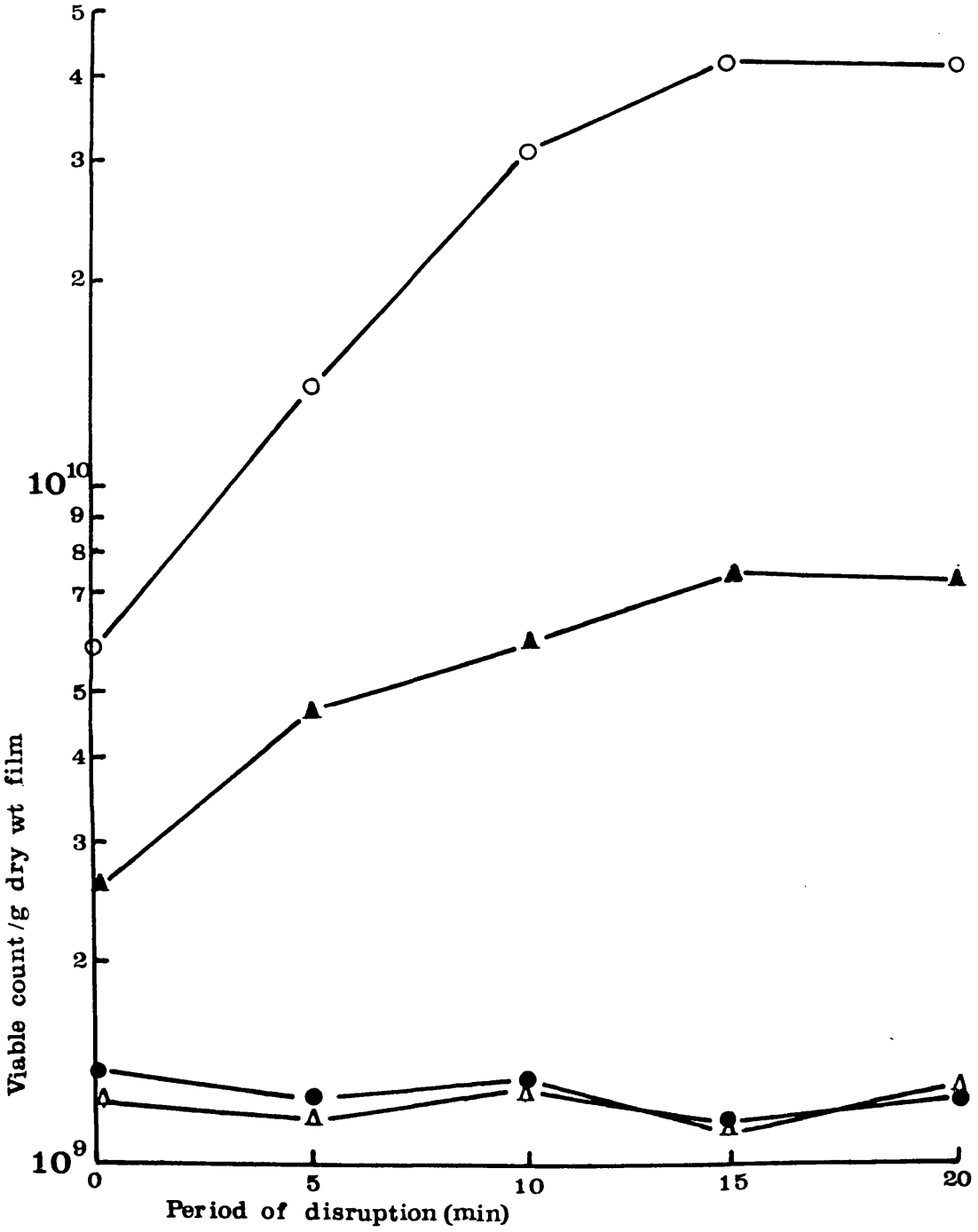

Fig. 7. The effect of shaking the film with glass beads on the recovery or microorganisms. Open circles, recovery on medium TA incubated at 25°; closed circles, recovery on medium TA incubated at 37° ; closed triangle, recovery on Nutrient Agar incubated at 25°; open triangles, recovery on Nutrient Agar incubated at 37°.

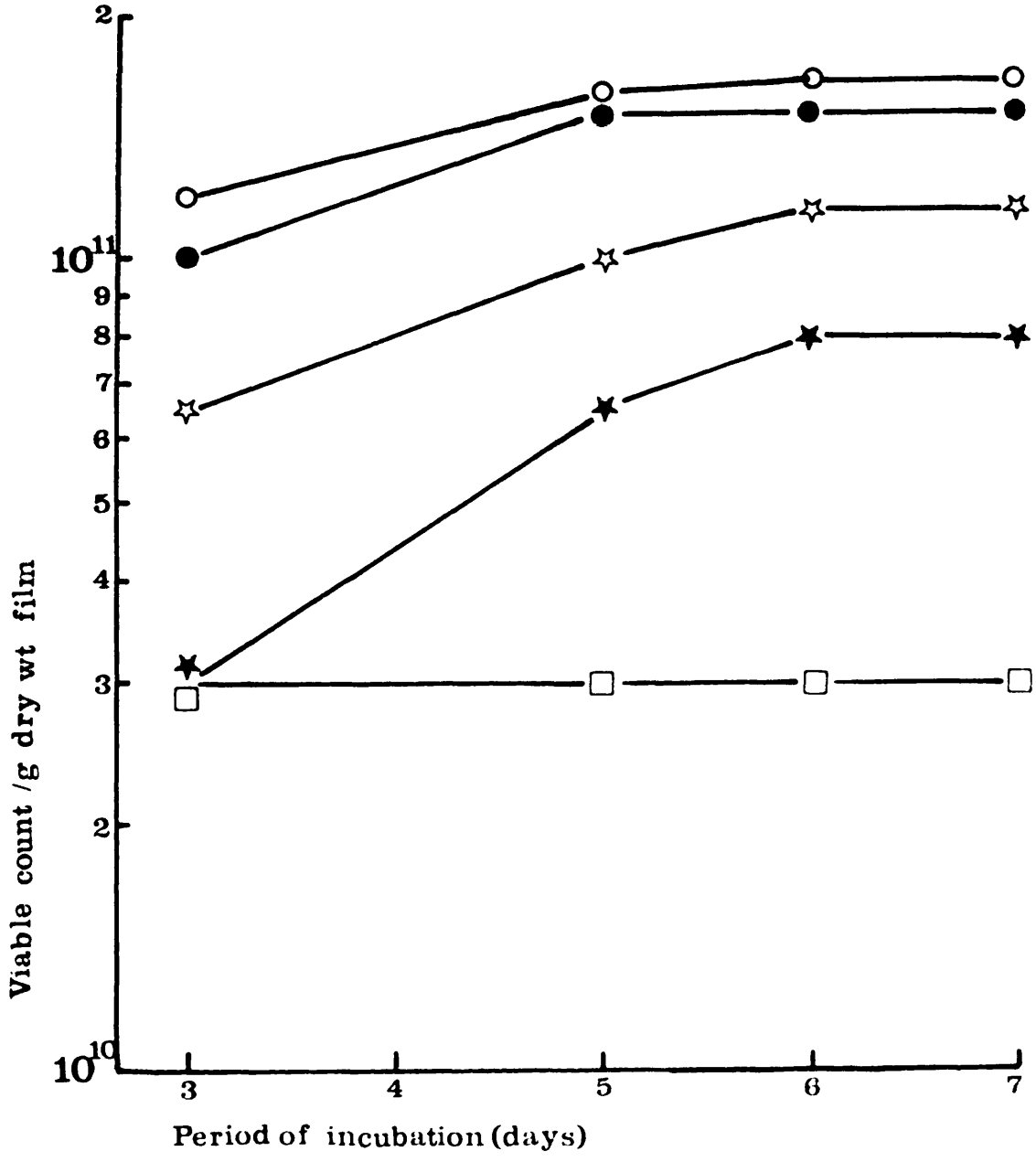

Fig. 8. The effect of the period of incubation at 25° on the recovery of organisms from a disrupted film. Open circles, medium TA; closed circles, medium TAG; open stars, Milk Agar; closed stars, Nutrient Agar; open squares, Blood Agar.

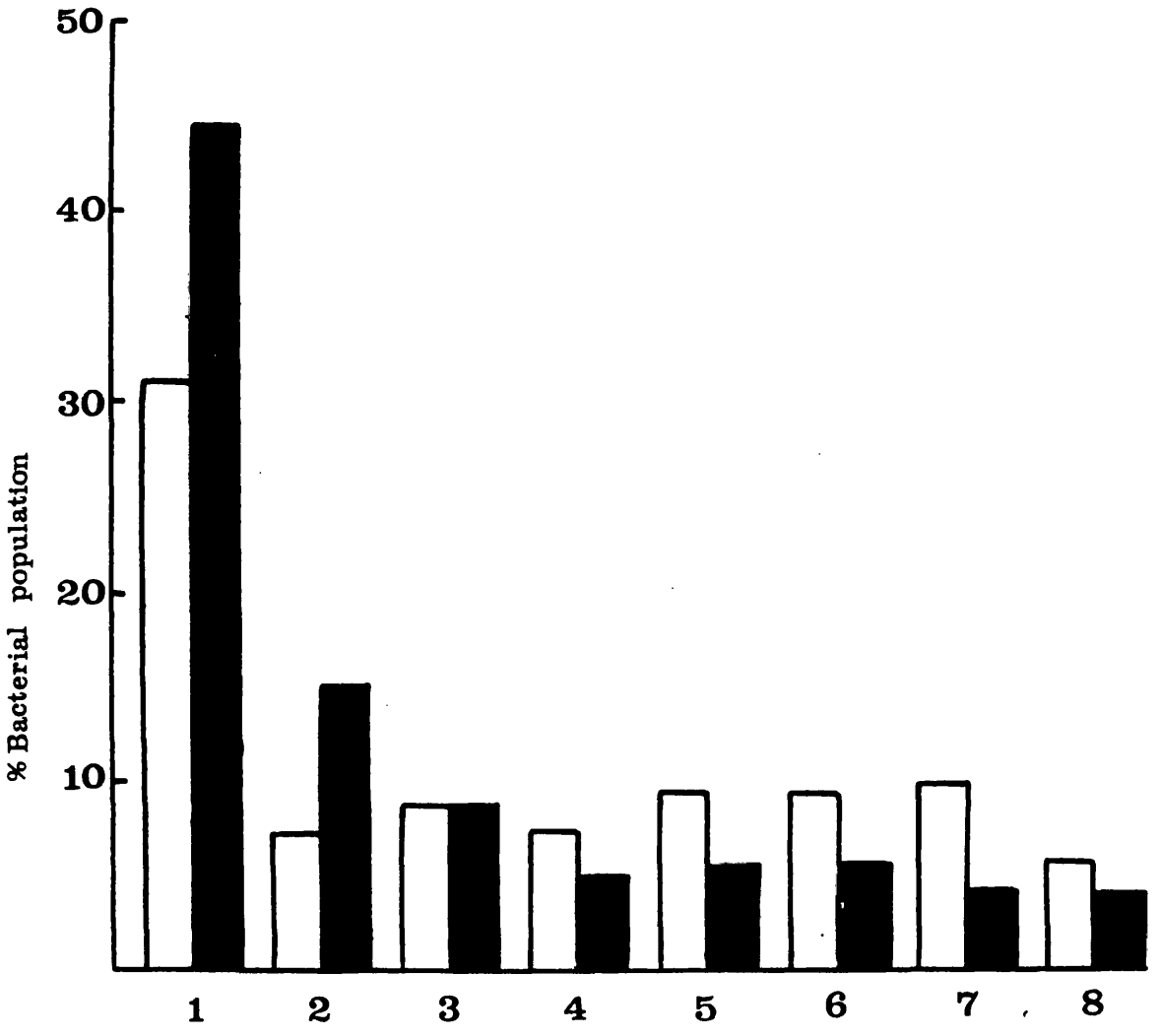

Fig. 9. Histogram of the microorganisms recovered from films developed by irrigation with domestic sewage. Open bars, young film; closed bars, mature film. Key to organisms; 1, Acinetobacter; 2, yellow-pigmented Gram-negative rods; 3, Corynebacteriaceae; 4, Alcaligenes; 5, Pseudomonas; 6, Enterobacteriaceae; 7, micrococci; 8, Bacillus.

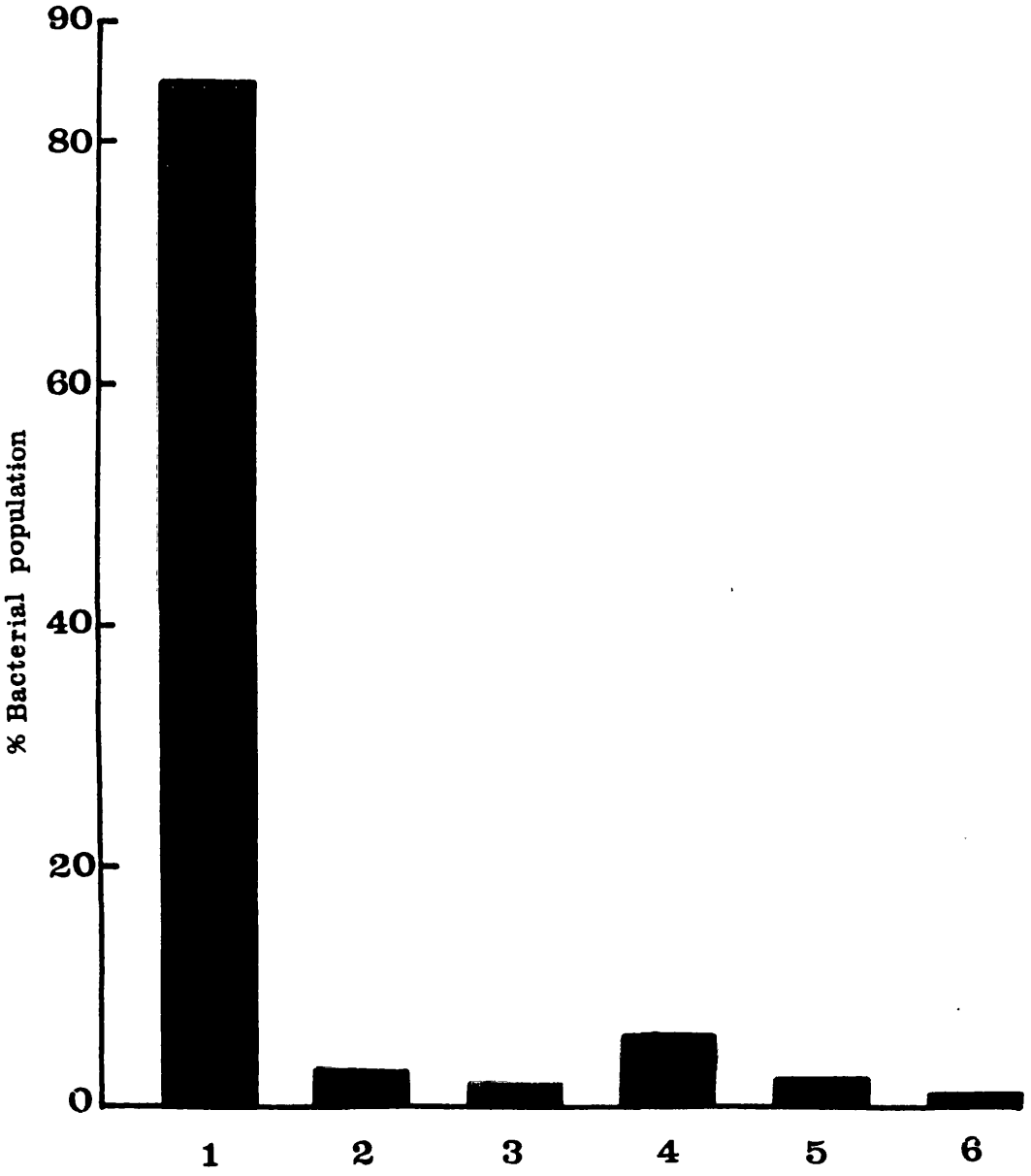

Fig. 10. Histogram of the microorganisms recovered from a film irrigated with dilute piggery waste (C 700 mg/l COD). Key to organisms; 1, Acinetobacter; 2, yellow-pigmented Gram-negative rods; 3, Pseudomonas ; 4, Enterobacteriaceae; 5, micrococci; 6, Bacillus.

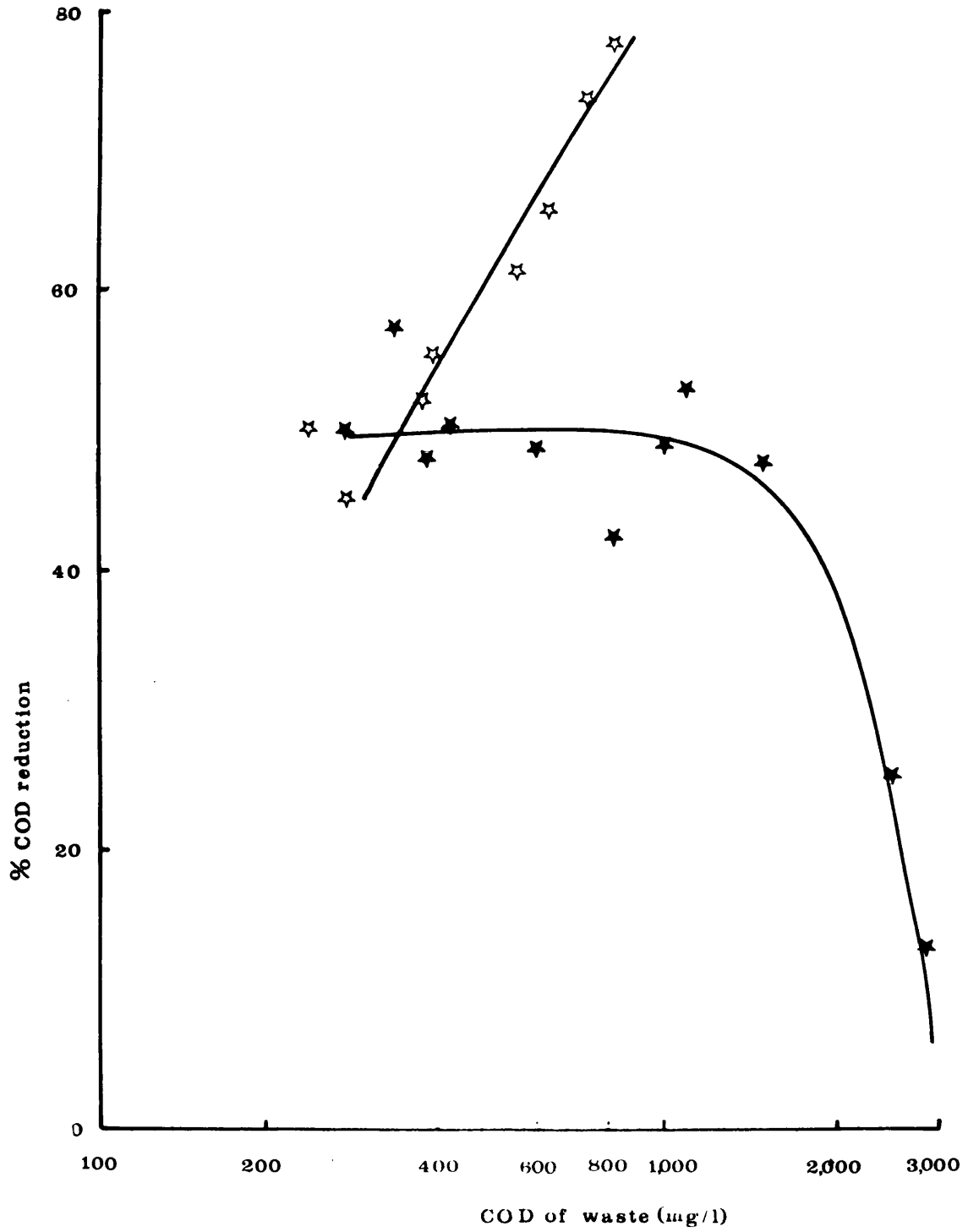

Fig. 11. Purification of wastes by the experimental trickling filters.

Open stars, domestic sewage; closed stars, diluted piggery waste.

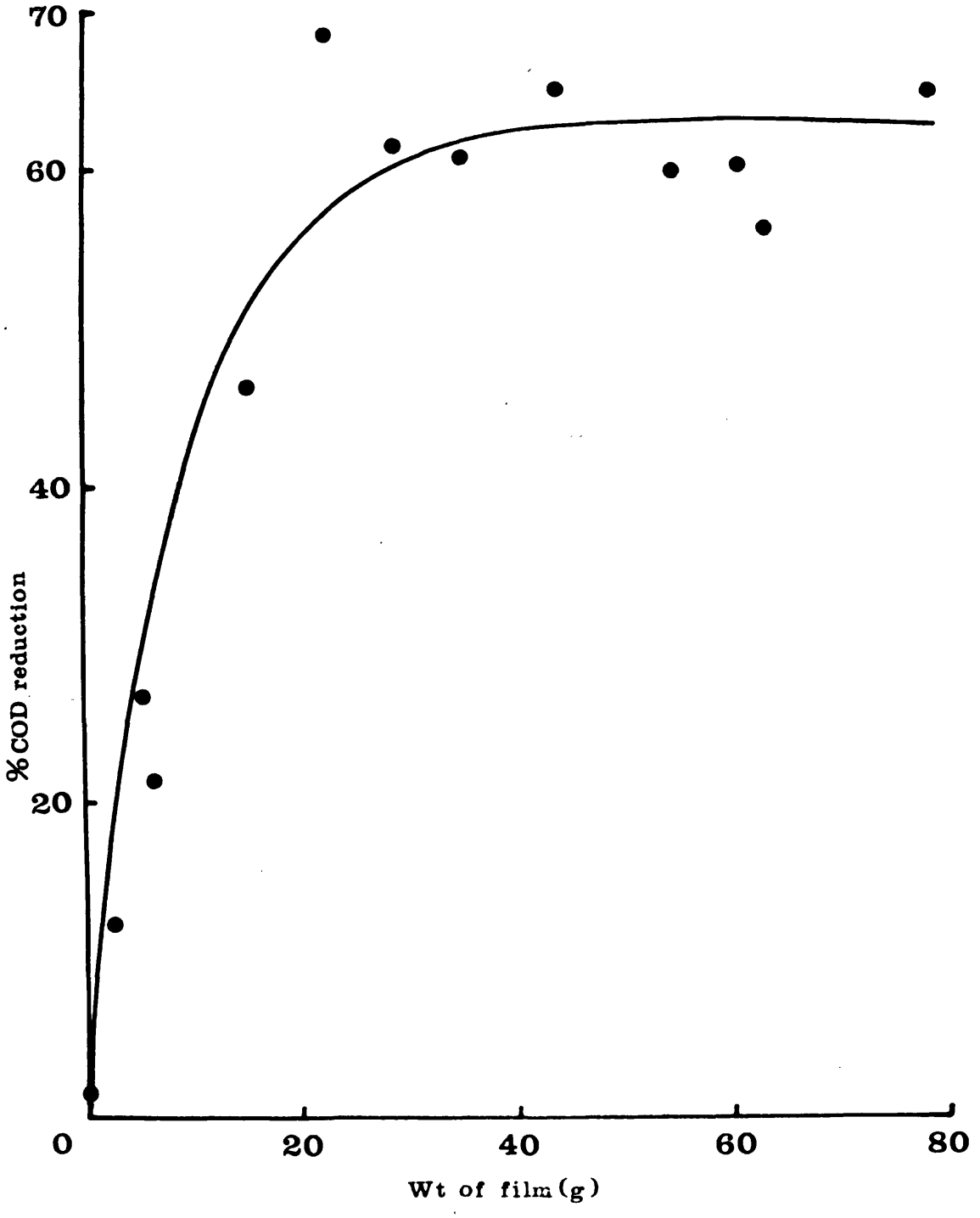

Fig. 12. Purification of domestic sewage by a developing biological film.

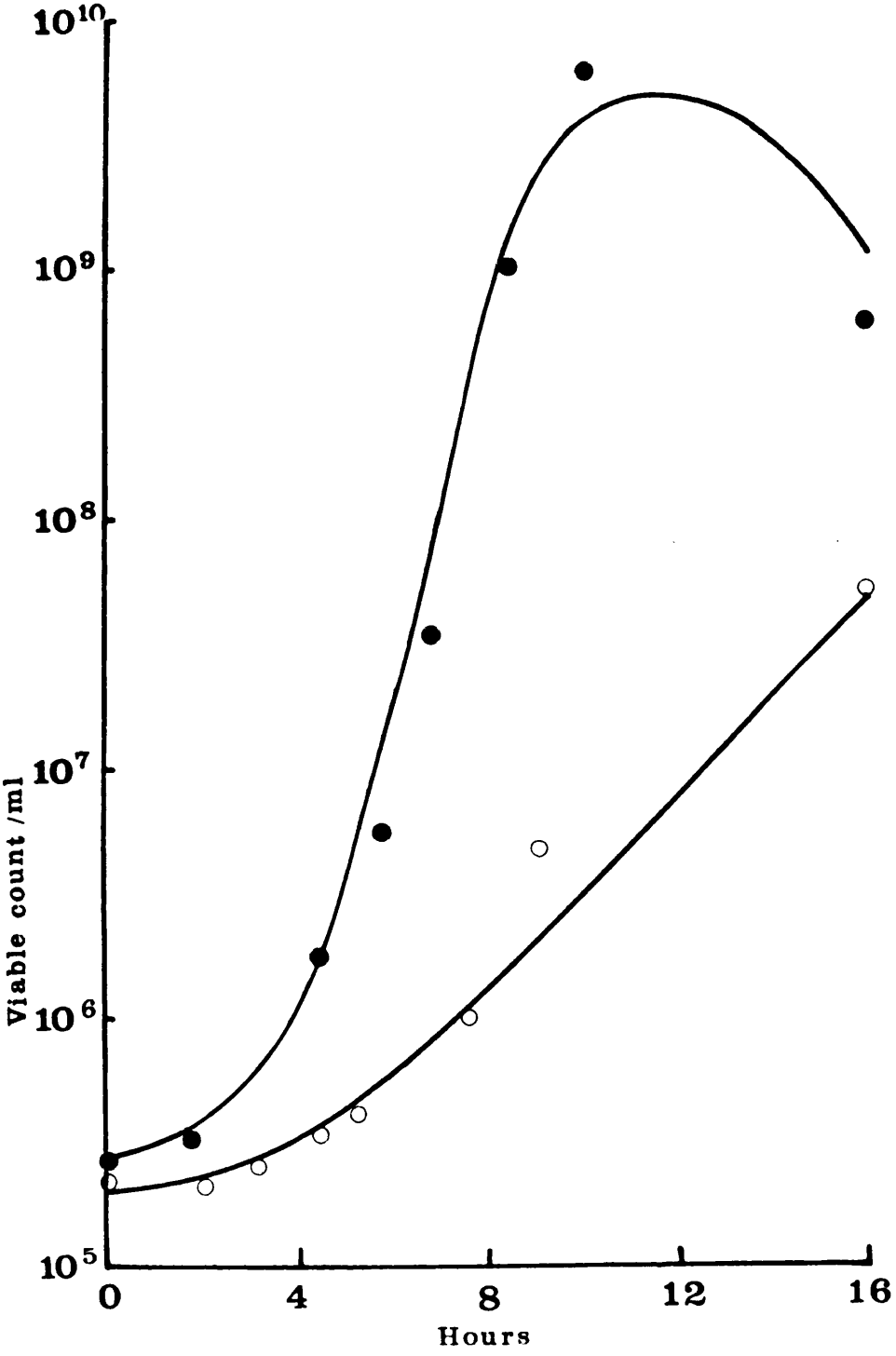

Fig. 13. Growth of Acinetobacter 4 and Pseudomonas putida in separate standing cultures of Synthetic Sewage medium. Closed circles, recovery of Pseudomonas on Glucose Mineral Salts Agar; open circles, recovery of Acinetobacter on medium TA.

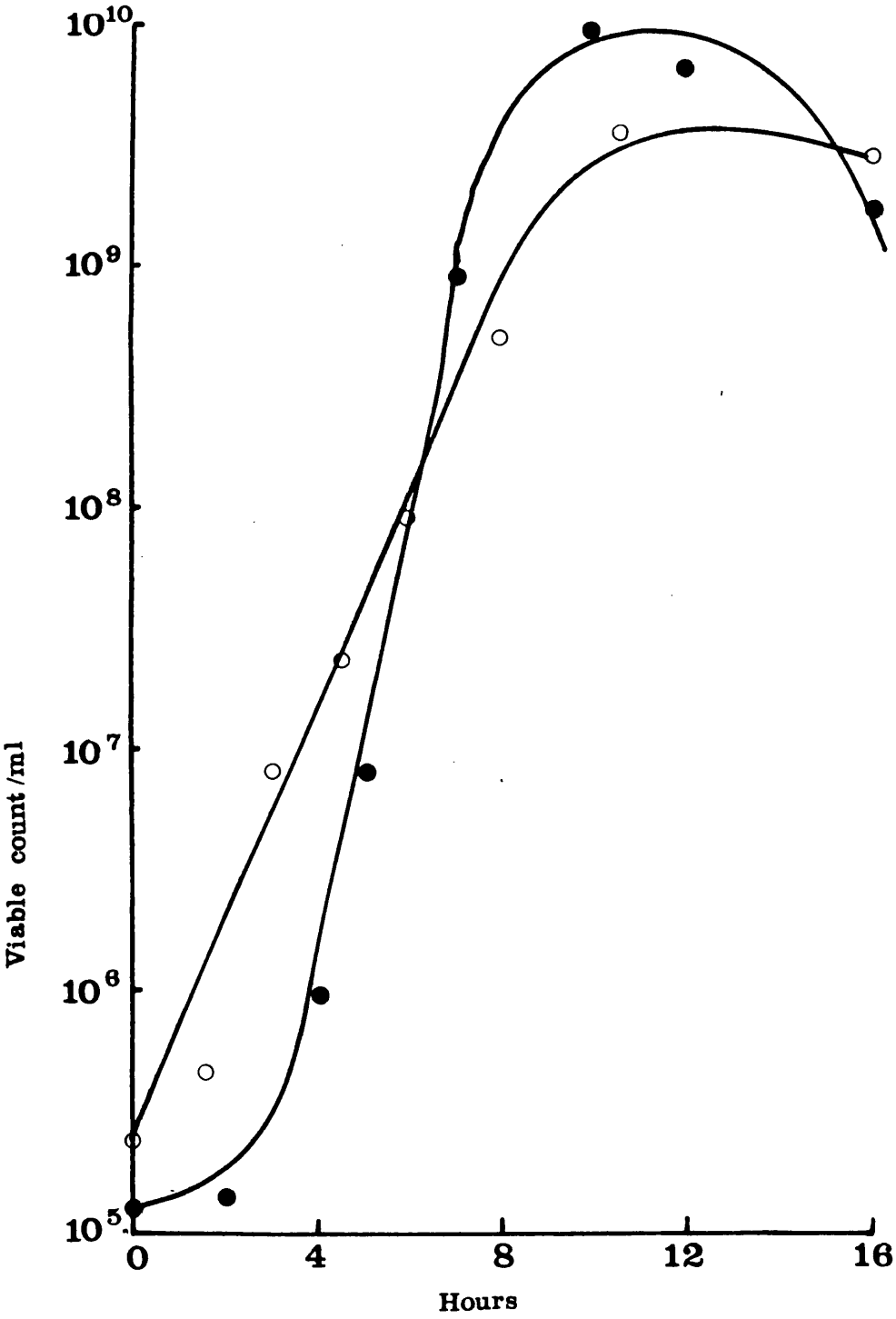

Fig. 14. Growth of Acinetobacter 4 and Pseudomonas putida in separate aerated cultures of Synthetic Sewage medium. Closed circles, recovery of Pseudomonas on Glucose Mineral Salts Agar; open circles, recovery of Acinetobacter on medium TA.

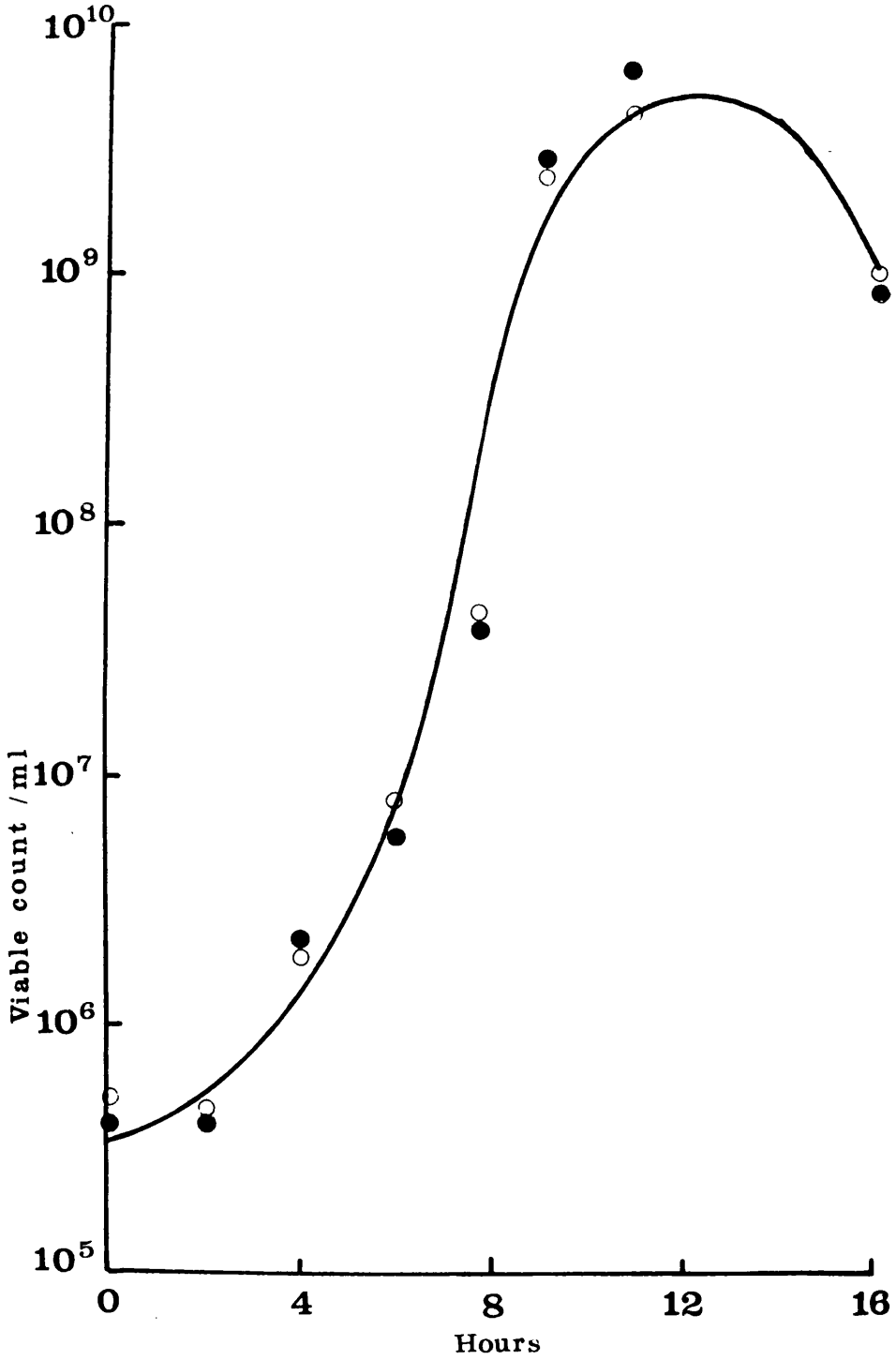

Fig. 15. Growth of Acinetobacter 4 and Pseudomonas putida together in standing cultures of Synthetic Sewage medium. Closed circles, organisms recovered on Glucose Mineral Salts Agar; open circles, organisms recovered on medium TA.

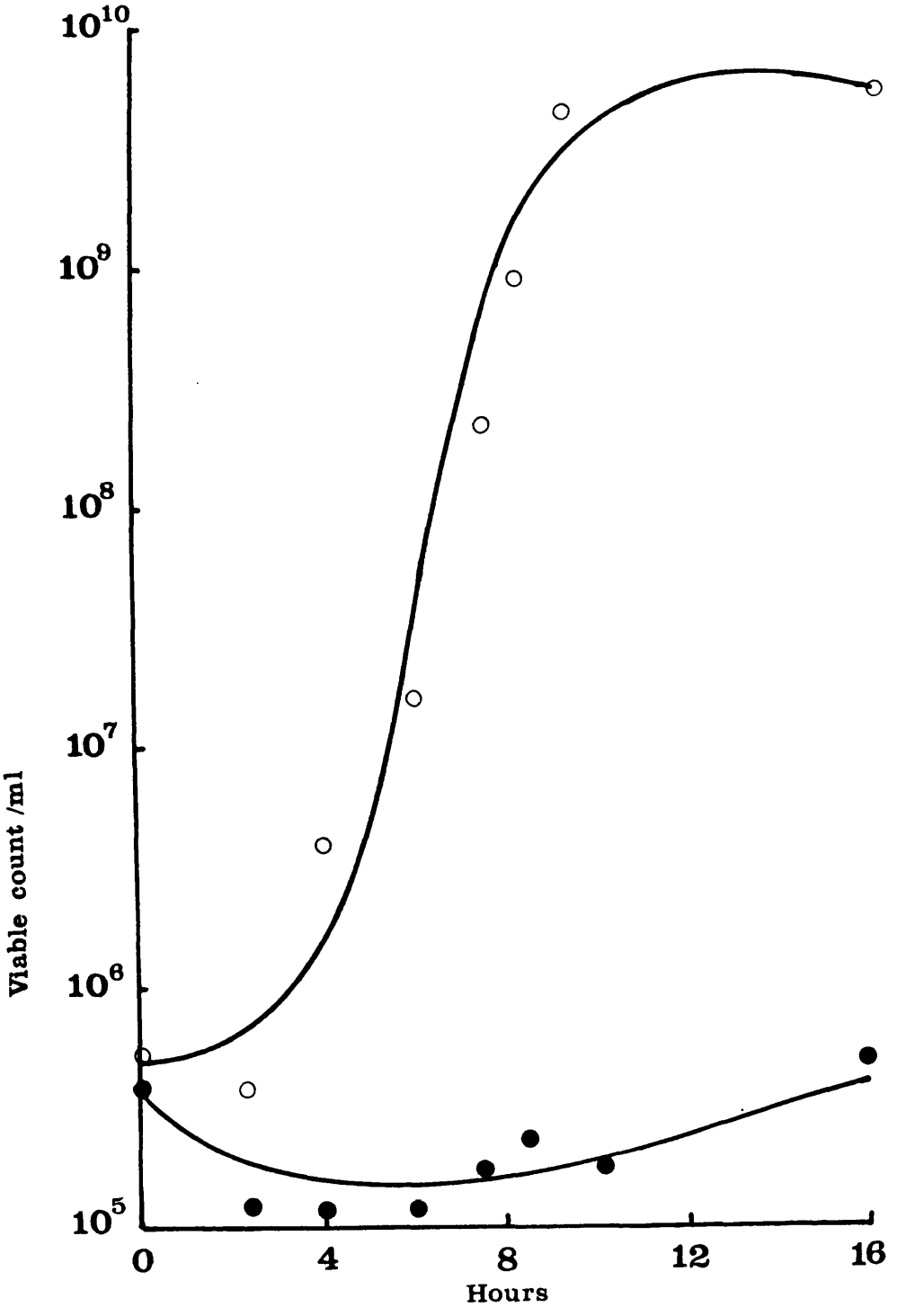

Fig. 16. Growth of Acinetobacter 4 and Pseudomonas putida together in aerated cultures of Synthetic Sewage medium. Closed circles, organisms recovered on Glucose Mineral Salts Agar; open circles, organisms recovered on medium TA.

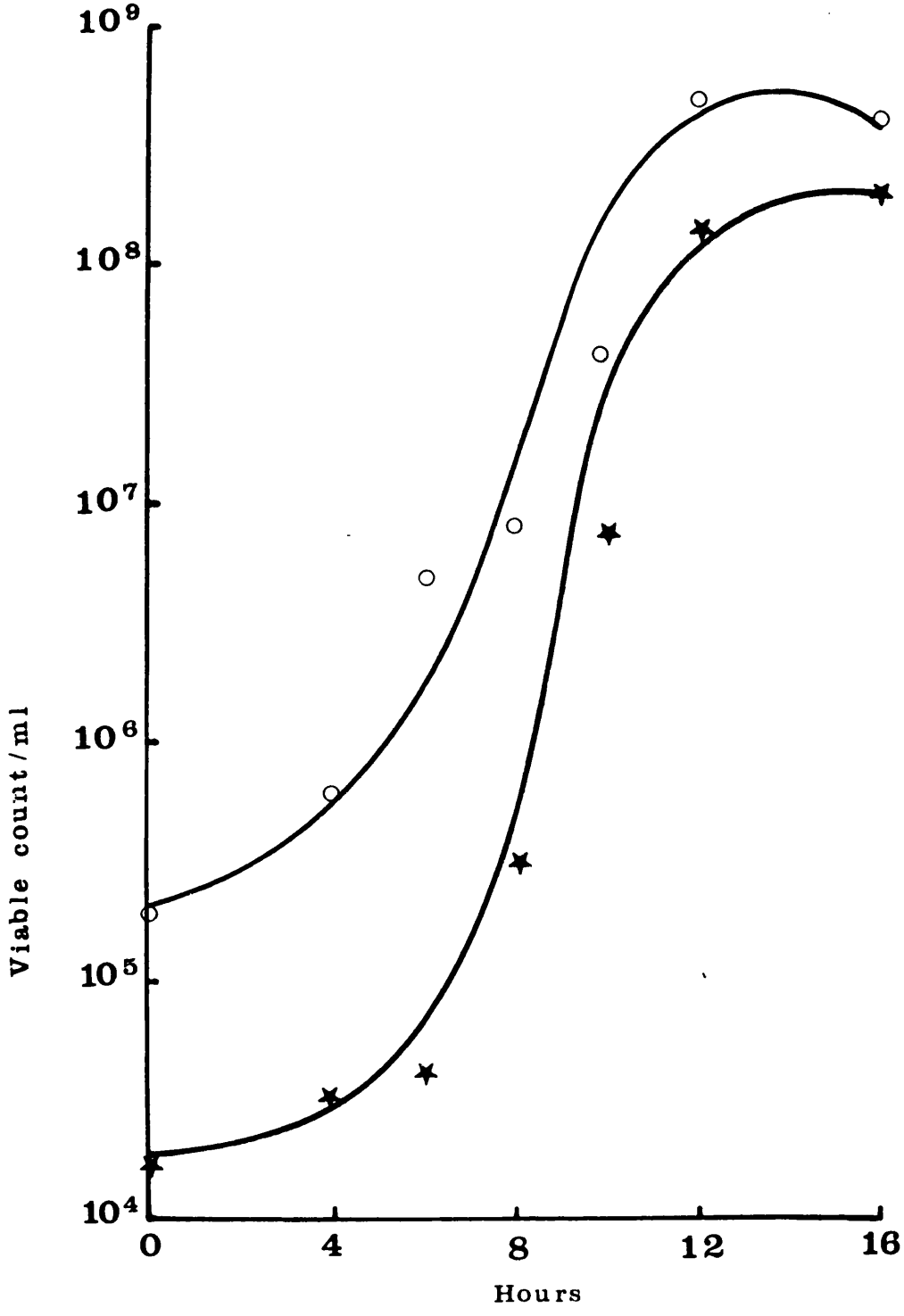

Fig. 17. Growth of Acinetobacter 4 and the yellow-pigmented rod in Synthetic Sewage medium. Open circles, Acinetobacter; closed stars, the yellow-pigmented rod.

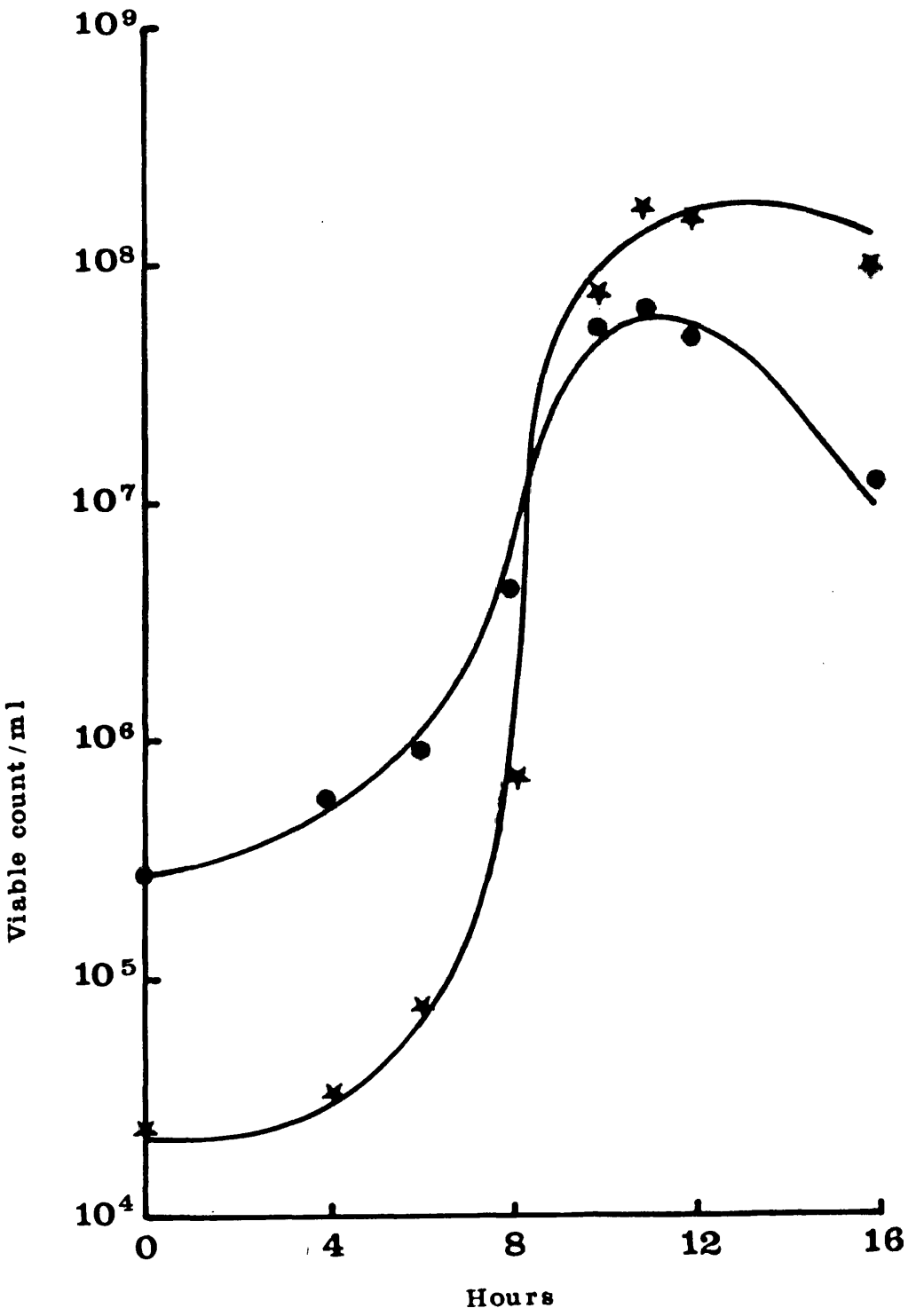

Fig. 18. Growth of Pseudomonas putida with the yellow-pigmented rod in Synthetic Sewage medium. Closed circles, Pseudomonas; closed stars, the yellow-pigmented rod.

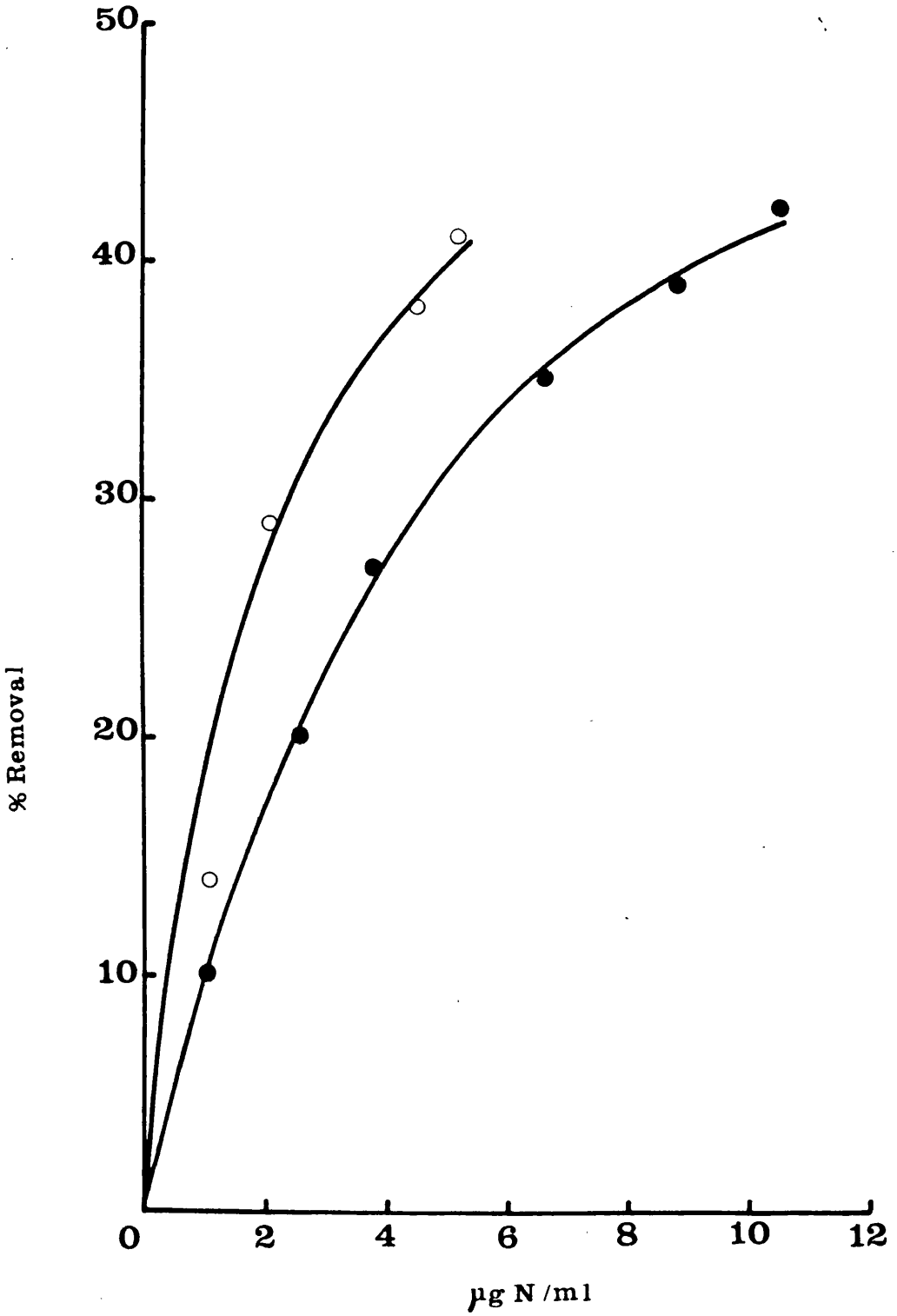

Fig. 19. Reduction of the COD of Synthetic Sewage medium (initial COD of the medium - 350 mg/l) as a result of the growth of Acinetobacter 4 and Pseudomonas putida. Open circles, Acinetobacter; closed circles, Pseudomonas.

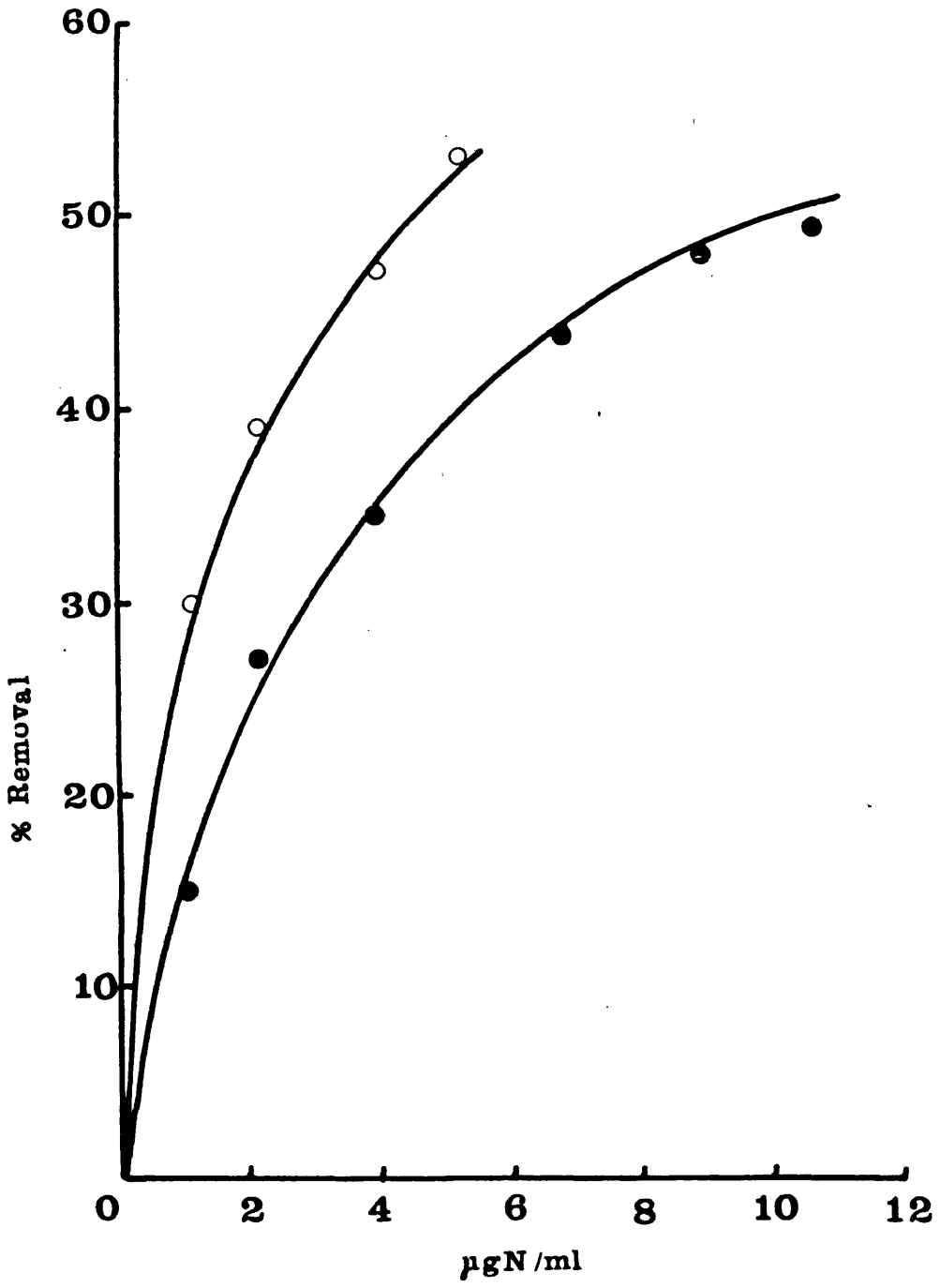

Fig. 20. Removal of protein from Synthetic Sewage medium (initial protein content of the medium - 375 µg/ml) as a result of the growth of Acinetobacter 4 and Pseudomonas putida. Open circles, Acinetobacter; closed circles, Pseudomonas.

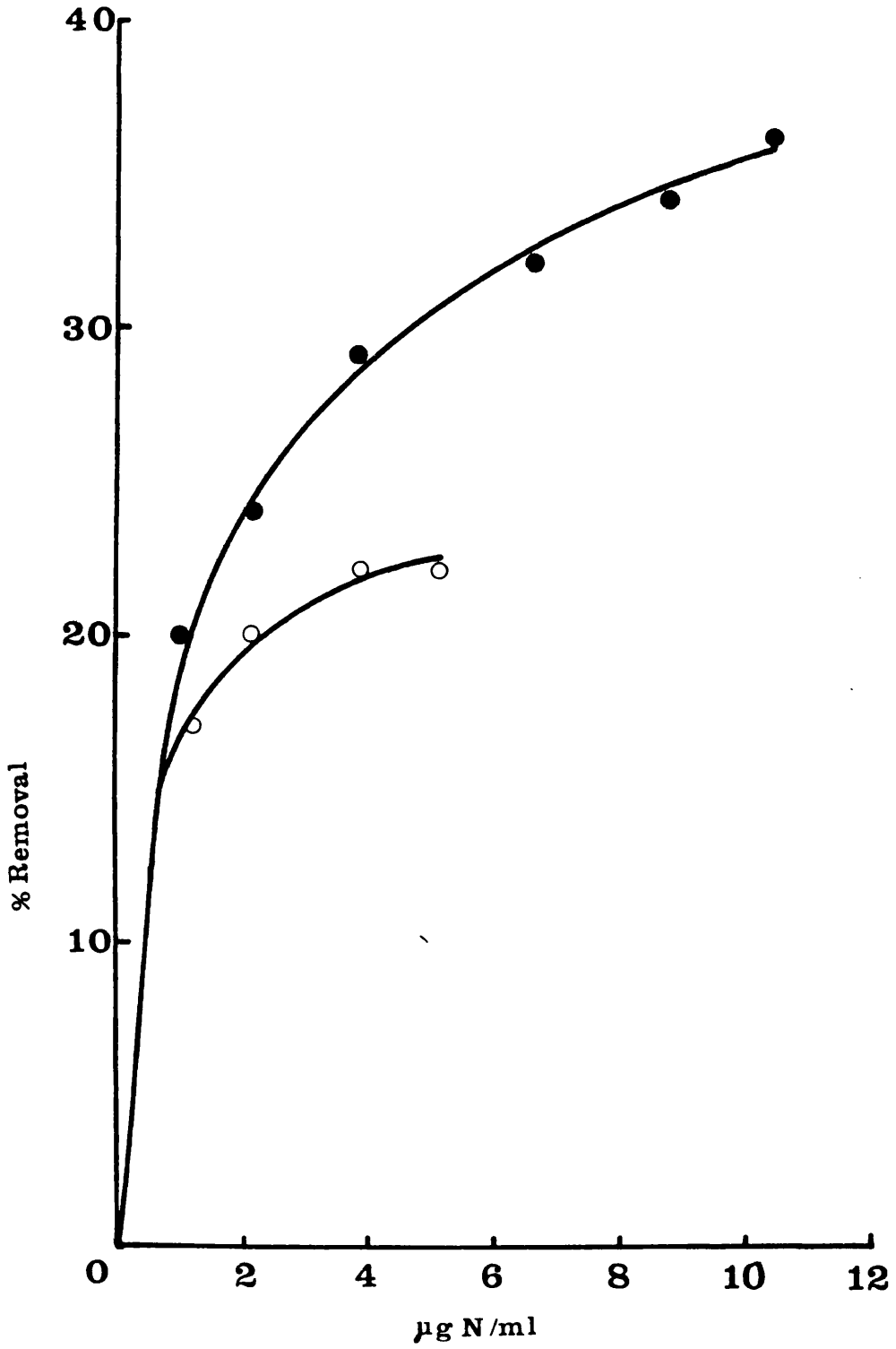

Fig. 21. Removal of carbohydrate from Synthetic Sewage medium (initial carbohydrate content - 91 µg/ml) as a result of the growth of Acinetobacter 4 and Pseudomonas putida. Open circles, Acinetobacter; closed circles, Pseudomonas.

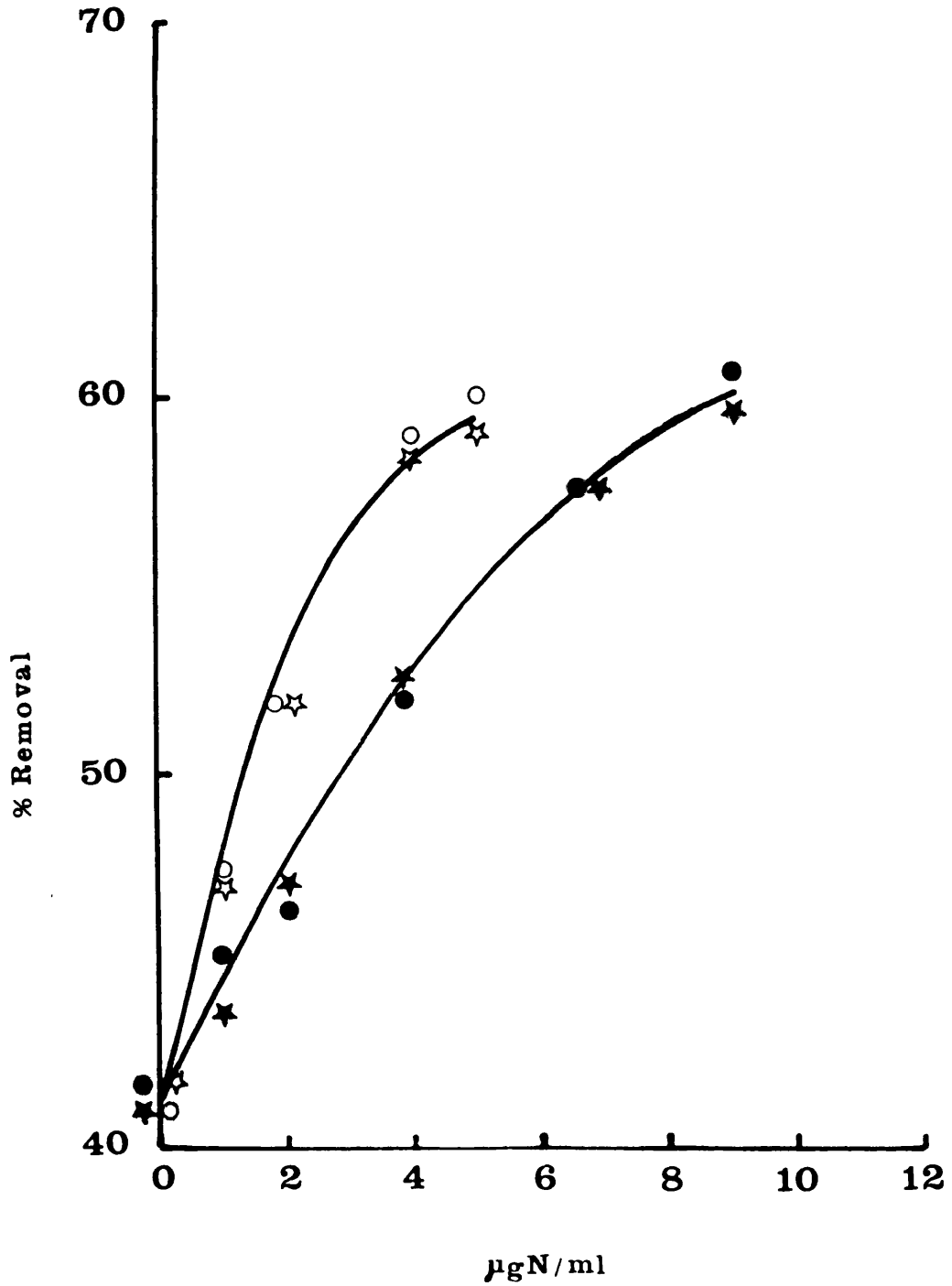

Fig. 22. Reduction of the COD of Synthetic Sewage culture filtrates (initial COD of the culture filtrate : 205 mg/l) as a result of the growth of Acinetobacter 4 and Pseudomonas putida. Open circles, Acinetobacter growing in its own culture filtrate; open stars, Acinetobacter growing in the culture filtrate from Pseudomonas; closed circles, Pseudomonas growing in its own culture filtrate; closed stars, Pseudomonas growing in the culture filtrate from Acinetobacter.

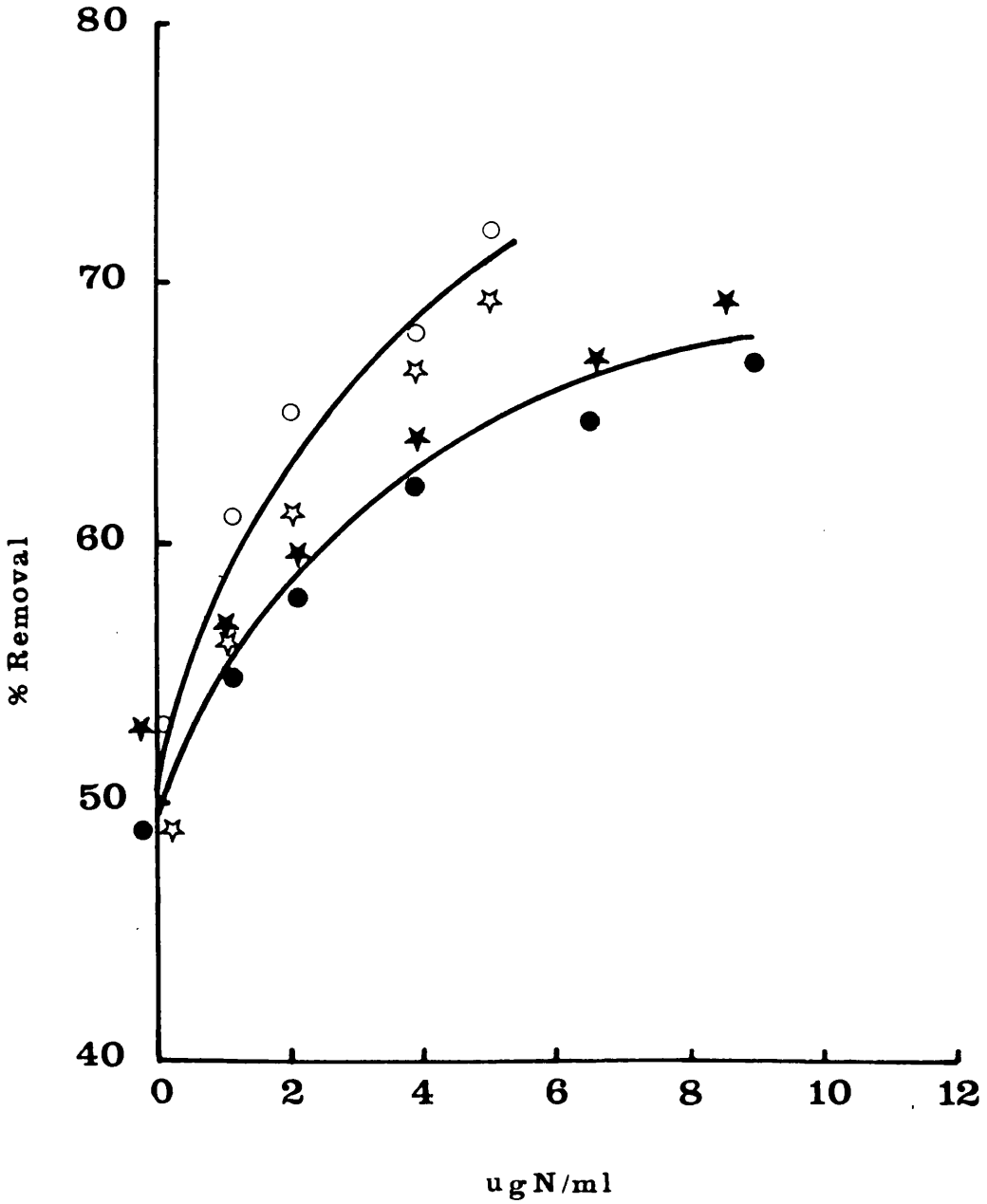

Fig. 23. Removal of protein from Synthetic Sewage culture filtrates (initial protein content : 170-180 µg/ml) as a result of the growth of Acinetobacter 4 and Pseudomonas putida. Open circles, Acinetobacter growing in its own culture filtrate; open stars, Acinetobacter growing in the culture filtrate from Pseudomonas; closed circles, Pseudomonas growing in its own culture filtrate; closed stars, Pseudomonas growing in the culture filtrate from Acinetobacter.

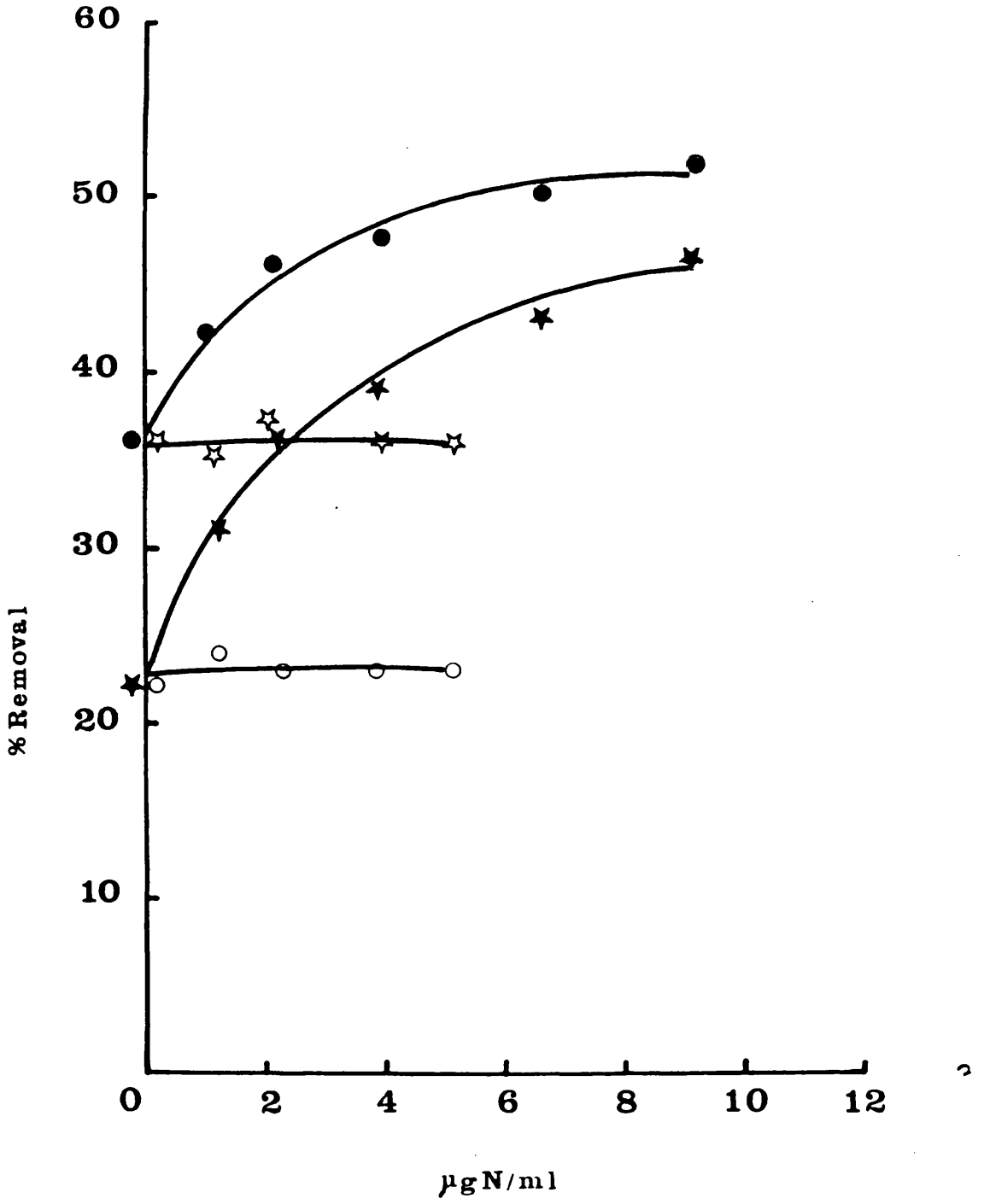

Fig. 24. Removal of carbohydrate from Synthetic Sewage culture filtrates (initial carbohydrate content : 50-70 µg/ml) as a result of the growth of Acinetobacter 4 and Pseudomonas putida. Open circles, Acinetobacter growing in its own culture filtrate; open stars, Acinetobacter growing in the culture filtrate from Pseudomonas; closed circles, Pseudomonas growing in its own culture filtrate; closed stars, Pseudomonas growing in the culture filtrate from Acinetobacter.

TABLES

TABLE 1

Some characteristics of piggery wastes and of settled domestic sewage

Suspended Solids (mg/l)	pH	Total Nitrogen (mg/l)	BOD (mg/l)	COD (mg/l)	Source
. .	7·1	46**	. .	300-700*	Settled sewage from Bath
7,000*	7·8	820**	. .	8-9,000*	Piggery Waste from Bath
32,000	. .	3,512	25,000	. .	Anon (1964)
. .	. .	2-9,000	1,275-13,260	. .	Baines (1964)
. .	. .	650-750	1,910-2,890	4,300-5,200	Ingens & Day (1966)
. .	8·7	. .	30,000	80,000	Scheltinga (1966)
4,500-8,000	. .	. .	930-10,800	. .	Smith & Hazen (1967)
. .	. .	5,500	33,000	. .	Anon (1968a)
7-16,000	7·4-8·8	. .	18,000-20,000	. .	Pontin & Baxter (1968)
3,614	. .	. .	1,156	3,903	Pratt et al. (1968)
. .	. .	. .	. .	406,000-536,000	Robinson et al. (1970)

* Anon (1965)

** Minari & Zilversmit (1963)

TABLE 2

Recovery of a tracer* from experimental trickling filters

Minutes after addition of tracer	Mean cumulative % recovery of tracer (0·3525 N sodium chloride)				
	Tube No.1	Tube No. 2	Tube No. 3	Tube No.4	Tube No. 5
1	6·35	7·90	8·50	6·45	5·55
2	20·00	30·30	24·55	33·20	30·50
3	58·35	55·00	50·85	55·00	45·45
5	72·85	66·10	69·25	70·60	60·45
7	83·85	75·30	79·95	78·85	73·55

* Na Cl

TABLE 3

Recovery of viable bacteria at 37° from the film developed on experimental trickling filters

Period of Incubation (days)	Viable Count /g				
	Medium TA	Medium TAG	Milk Agar	Nutrient Agar	Blood Agar
2	$1{\cdot}15 \times 10^9$	$1{\cdot}08 \times 10^9$	$1{\cdot}18 \times 10^9$	$1{\cdot}12 \times 10^9$	$1{\cdot}2 \times 10^9$
3	$1{\cdot}15 \times 10^9$	$1{\cdot}08 \times 10^9$	$1{\cdot}18 \times 10^9$	$1{\cdot}12 \times 10^9$	$1{\cdot}2 \times 10^9$
4	$1{\cdot}15 \times 10^9$	$1{\cdot}08 \times 10^9$	$1{\cdot}18 \times 10^9$	$1{\cdot}12 \times 10^9$	$1{\cdot}2 \times 10^9$

TABLE 4

A summary of the tests used for the identification at generic level of organisms recovered from trickling filters

Medium	Observation
Medium TA - streaked to give isolated colonies	Appearance of colonies, Gram Stain, Morphology, Oxidase test
0·25% (w/v) Peptone water	Motility - hanging drop preparation examined by phase contrast microscopy
Hugh & Leifsons (1953) medium containing 1% (w/v) glucose	Breakdown of carbohydrate, viz oxidative, acid formed in the presence of molecular oxygen ; fermentative, acid formed both in the presence and absence of molecular oxygen

TABLE 5

Diagnostic table for organisms recovered from trickling filters

Property	Acinetobacter	Alcaligenes	Pseudomonas	Yellow rods	Corynebacteriaceae	Bacillus	Micrococci	Enterobacteriaceae
Reaction to Gram's Stain	-/v	-	-	-	+	+	+	-
Morphology	CR	R	R	R	Co	R	C	R
Pigmentation	-	-	-/BG	Y	-/Y	-	-/Y	-
Oxidase	-	-	+	+	-	-	-	-
Motility	-	+	+	-	-	+	-	+/-
Action in Glucose	-	-	O	+	-	O/F	O	F

+, positive ; -, negative or no change; v, variable : R, rod; CR, coccoid rod;

C, coccus; Co. coryneform; BG, blue-green; Y, yellow; O, oxidative ;

F, fermentative.

TABLE 6

Properties used in the diagnosis of the three organisms representing the major groups of bacteria isolated from trickling filters

Property	Acinetobacter (Phenon 4)	Pseudomonas putida	Yellow-pigmented rod
Reaction to Gram's stain	-/v	-	-
Morphology	CR	R	R
Pigmentation of colony	-	-	Y
Motility	-	+	-
Oxidase	-	+	-
Gelatin hydrolysis	-	-	+
Spreading Growth	-	-	-
Growth at 37°	-	-	-
Arginine hydrolysis	-	+	-
Production of Fluorescent pigments	. .	+	. .
Production of Phenazine	. .	-	. .
Oxidative acid from Carbohydrates	-	+	-

+, positive ; -, negative or no change ; V, variable; CR, coccoid rod;

R, rod; Y, yellow

TABLE 7

The mean generation times of Pseudomonas putida and Acinetobacter 4 in standing and aerated cultures of synthetic sewage medium

Culture	Pseudomonas putida	Acinetobacter 4
Standing	27·0 min	94·8 min
Aerated	26·4 min	38·4 min

Table 8. Principal bacterial genera isolated from waste treatment processes

	Sources *						
	Activated Sludge						Trickling Filters
	A	B	C	D	E	F	G
Group 1							
Achromobacter	P	P	-	P	P	D	D
Alcaligenes	P	D	-	P	D	-	D
Flavobacterium	P	D	-	P	D	P	D
Pseudomonas	D	P	D	P	P	D	D
Group 11							
Bacillus	P	P	P	P	-	P	P
Micrococcus	P	P	P	P	P	P	-
Group 111							
Comamonas	-	-	-	D	-	-	-
Corynebacteriaceae	-	-	-	P	P	D	-
Zoogloea	P	-	-	D	-	-	D
Group 1V							
Aerobacter	P	-	-	P	-	-	-
Bacterium	P	-	P	-	-	-	-
Microbacterium	P	P	-	-	-	-	-
Nocordia	P	-	-	-	-	-	-
Sarcina	-	-	P	-	-	-	-
Spirillum	-	-	-	P	-	-	-

P, present; D, present in large numbers

* A, McKinney & Weichlein (1953); B, Jasewicz & Porges (1956) ; C, Rogovskaya & Lazareva (1959); D, Dias & Bhat (1964); E, Van Gils (1964) ; F, Adamse (1966); G, James (1964)

Table 9. Bacterial associations reported in waste treatment processes

	Sources *						
	Activated Sludge						Trickling Filters
	A	B	C	D	E	F	G
Pseudomonads	D	D	D	P	P	D	D
Achromobacters	P	-	P	D	D	D	D
Flavobacteria	P	-	P	D	D	P	D
Coryneforms	-	-	P	-	P	D	P

P, present; D, present in large numbers

* A, McKinney & Weichlein (1953); B, Jasewics & Porges (1956);
C, Rogovskaya & Lazareva (1959); D, Dias & Bhat (1964);
E, Van Gils (1964); F, Adamse (1966); G, James (1964).

DISCUSSION

TRICKLING FILTERS

Traditionally trickling filters are beds 6 ft deep packed with graded pieces of clinker, broken rock, gravel or slag (the filter medium). Although solids present in the wastes may be trapped in the filter bed, mechanical filtration is not an important aspect of purification. The filter medium exists to support microorganisms which develop as a film on its surfaces and which clarify and oxidise the waste. As such its requirements are that it should be inert of sound mechanical strength, possess an extensive and exposed surface for growth of film, and have adequate void spaces to allow for film accumulation, free passage of waste and access of air.

In the treatment of wastes at high organic loadings, conventional stone packed filter beds have a tendency towards excess accumulation of film and subsequent occlusion of the void spaces in the filter medium. Geometrically-ordered plastic filter media have been developed to provide a solution to this problem. They have a surface area comparable to that of conventional media but a much larger proportion of void space and a low bulk density. Continuous and relatively wide gaps permit the unrestricted flow of liquid and entrained solids over the surfaces, and the largely vertical arrangement of the plastic packings discourages the accumulation of thick layers of film because they tend to slide off under their own weight.

In 1882 it was suggested by Warrington that man-made filter beds might have certain advantages over land treatment for the purification of sewage (Bruce, 1969), and they were in use in the

United Kingdom by 1889 (Stanbridge, 1954). Their general characteristics (i.e. tolerance to variation in the volume and composition of the applied wastes, simplicity of operation and control, etc.) have led to their becoming the most widespread and largest application of sewage treatment (Bruce, 1969). Currently plastic filter media, of which there are several proprietary designs, are being widely used for partial treatment of industrial wastes at high organic and/or hydraulic loadings.

Experimental problems

Investigations into the biology and the activities of trickling filters have been hampered because of difficulties in obtaining representative samples of the film and the effluent from within the depths of the filter bed. Although specially constructed filter beds in which chemical and bacteriological samples should be obtained at different depths without disturbing the remainder of the bed have been available to some workers (Velz, 1948; James, 1964) most have used model systems in their studies.

The requirements of a model system for studying trickling filters are that a film should develop on a carrier, (1) whose surface area and weight can be accurately determined, (2) which does not clog as a result of excess accumulation of film, and (3) from which the complete film can be removed easily. Some workers have confined themselves to miniature and small scale trickling filters. To obtain samples from varying depths, Butterfield & Wattie (1941) used a stone-packed column (30 x 30 x 72 in) which was constructed in three sections, and in their study of trickling filters colonized with pure cultures they used stone-packed glass tubes (length 30 in, inside diameter 2 in). In other studies, unglazed tiles have been positioned in channels carrying sewage (Heuhelekian & Crosby, 1956) and vertical

wire screens have been irrigated with settled sewage (Schulze, 1960; Harkness, 1966). Maier (1968) devised a system consisting of a screen of fibre glass wire (0·028 in diameter, woven 5 x 7 strands/cm) cemented to the upper surface of an inclined flat plastic plate (11 cm wide, 60 cm long). The purpose of the fibre glass screen was to act as a framework for film growth, and the thickness of the film could be controlled to about the thickness of the framework by scraping off any excess with a knife. However in their apparatus a precision engineered weir was required to ensure a uniform and laminarflow of the waste over the whole surface of the plastic plate. The type of system used in the present study was based on an apparatus (Gloyna, Comstock & Renn, 1952) in which, as a result of irrigation with wastes, microbial films develop in the inner surfaces of inclined rotating Perspex tubes. In addition to fulfilling the requirements of a model system, the rotating tubes are more compact and more easily accessible for maintenance than other small scale or model systems of equivalent capacity. The work of Gloyna et al. (1952) served only to indicate the usefulness of this type of apparatus in laboratory experiments. Subsequently, rotating tubes have been used (Tomlinson & Snaddon, 1966) to study the effects of weight and thickness of the film on the purification of domestic sewage. Part of the present study was concerned with the development of films on the rotating tubes and the relationship of the efficiency of waste purification to the weight of film and to the strength of the waste.

The film

It has been claimed (Gloyna et al., 1952) that the microbial films which develop in rotating tubes function like those on stones of conventional filter beds and that accretion of films (Tomlinson & Snaddon, 1966) is exponential with time when the tubes are irrigated with weak domestic sewage, findings which have been corroborated in the

present study (Fig. 5). Film accruing as a result of irrigation with wastes containing high concentrations of organic matter have been described as watery and having a tendency to slough off more easily than those developed on a weaker waste (Heukelekian & Crosby, 1956; Saunders, 1966). In the present study it was impossible to initiate film development, except as sparse watery patches, with strong wastes (> 2,000 ug/l COD) coming from intensively house pigs (Fig. 5). Increase in the amount of film within a trickling filter is a product of cell growth and deposition of coagulated solids from the waste. Film accretion is not confined to the initial period of irrigation, but if growth should continue without restriction it would lead to the interstices of the medium becoming closed, thus impeding the passage of the waste and respiratory gases. In practice, however, the amount of film present on the filter medium is controlled by mechanisms which include predation by protozoa, physical removal of loose film and debris by liquid flow, endogenous respiration and autolytic decomposition. Accumulation of film in the present study, as a result of irrigation with domestic sewage (Fig. 6, Appendix 1) was never as great as it was during the first few days, yet protozoa, which have often been considered (Hawkes, 1963) as the principal agents of film control, were never detected.

During the first few days of irrigation of rotating tubes the efficiency of purification (in terms of reduction of COD) of domestic sewage was proportional to the amount of film present (Fig. 12), but any increase beyond 20g/tube did not affect purification. Similar results were obtained by Tomlinson & Snaddon (1966), the minimum amount of film for efficient purification of domestic sewage by rotating tubes was found to be 18 g/tube. It was suggested (Tomlinson & Snaddon, 1966) that a minimum thickness of film (C. 0·15 mm) was

required for purification. However it would appear from their synonymous use (Tomlinson & Snaddon, 1966) that mean thickness of film was calculated from the weight. As during the first few days of irrigation growth was graduated from the inlet to the outlet ends of the tube, it would appear that unless the surface area of film was taken into account their estimates of mean thickness during this period must have been low. Purification was only proportional to the weight of film when there was insufficient film to clothe all of the inner surface of the tube. Therefore it is tempting to suggest that purification is a function of the surface area of film over which the waste flows rather than the weight or the thickness of the film, and this has been demonstrated experimentally with a conventional filter bed (Truesdale, Wilkinson & Jones, 1962).

Kinetics of purification

There have been many attempts to derive expressions whereby the performance of filter beds can be predicted from the parameters of hydraulic and organic loadings. Attempts have been made to describe the rate of removal of organic matter from the waste by equations analogous to those use in chemical kinetics, the biological nature of the film being largely ignored. Yet purification by trickling filters is a complex application of continuous microbial culture in which the nutrient medium (waste) flows through a static space in which microorganisms grow on a fixed carrier.

The complex nature of wastes and methods of monitoring the concentration of biodegradable matter therein, are major problems in devising mathematical models of waste treatment processes. For example future work may reveal that dissolved matter may be removed from the waste in a different manner from suspended matter.

In both categories, some compounds (the recalcitrant compounds) may be resistant to biodegradation, yet it is only the biodegradable portion that can be included in mathematical formulations. In most models either BOD or COD is used as a measure of the strength of the waste but neither of these methods distinguishes between the biodegradable and recalcitrant fractions. The object of measuring the BOD of a waste is to determine the effect that the waste will have on the oxygen resources of a natural body of water that receives the waste. The COD provides a measure of the oxygen equivalent to that portion of the organic matter in the waste which is susceptible to oxidation by a strong chemical oxidant. These methods provide only parameters of over-all pollution capability, and neither can be regarded as satisfactory for devising and testing theoretical considerations of waste treatment processes.

Of the kinetic formulations it is considered that the most valid are those which have been derived from experiments done at constant hydraulic loading. This is because it is not normally possible to vary the hydraulic load on a filter bed without affecting the organic load. It is possible to introduce the hydraulic load as a modification (Levine, Luebbers, Galligan & Vaughn, 1936; Schulze, 1960) but the primary consideration of theoretical models must be the effect of varying organic loads whilst all other factors are held constant.

The theory of Velz (1948) has frequently been considered as a basis for formulations applicable to all filter beds, it has been stated as : the rate of extraction of organic matter per interval of depth of a biological bed is proportional to the remaining concentration of organic matter measured in terms of its removability. It was considered that the soluble organic matter comprised the recalcitrant fraction, therefore purification was dependant upon the proportion of the organic matter in suspended and colloidal form as distinct from that in true solution

(Velz, 1948). However it has been shown that on average the organic carbon in settled sewage is equally divided between dissolved and suspended matter (Painter & Viney,1959) and that 80% removal of soluble organic carbon can be achieved in experimental trickling filters (Tomlinson & Snaddon, 1966).

As the waste passes over the filter medium, soluble and suspended organic matter is transferred to the stationary film. If the rate of reaction (removal of organic matter) is determined (Velz, 1948) by the concentration o f one reactant (organic matter in the waste), the kinetics of removal of pulluting matter by trickling filters can be considered to approximate to a first order reaction (Eden, Brendish & Harvey, 1966). If a is the concentration of the reactant at the start of an experiment (i.e. at t = o), and if x is the amount decomposed after time, t, then (a-x) is the concentration of the remaining reactant at time, t, and dx/dt is the rate at which the substance is decomposing (i.e. the rate of reaction). The law of mass action states that the rate is proportional to the active mass of the single reactant giving the first order equation :

$$dx/dt = k\,(a-x).$$

k is the proportionality constant and a is a constant for a given experiment. The variables x and t can be separated to give an equation which relates a small increment in x to a small increment in t :

$$dx/(a-x) = kdt.$$

Both sides of the equation are integrated between corresponding limits. At t=o, the start of measurements, x=o and if x=x, when t=t,

$$\int_0^x dx/(a-x) = k \int_0^t dt$$

giving - $$\ln (a-x) + \ln (a) = kt$$

This integrated equation applies to all values of t and the corresponding values of x, and as the subscripts to x, and t, may be dropped. Converting natural to common logarithms and rearranging :

$$\log (a-x) = \log (a) - \frac{k}{2 \cdot 3} t.$$

Therefore if a series of measurements of x at different times, t, are made and log (a-x) is plotted as ordinate against t as abscissa, a straight line should result of slope - k/2·3 and intercept at t = o of log (a). If a straight line is obtained, it is proof that the reaction obeys the first order law.

In a homogeneous, completely mixed, reaction it is easy to obtain samples and measure the rate of decomposition of the substrate. However it is impossible to obtain samples at time intervals from trickling filters but it is possible to substitute depth for time in the first order equation. This is because purification occurs most repidly in the upper regions of the filter (Velz, 1948; Bruce, 1969) where the organic matter in the waste is most concentrated, and because it has been shown (Bloodgood, Teletzke & Pohland, 1959) that the contact time between liquid waste and the film is directly proportional to the depth of the filter bed. This was done with samples from a specially constructed filter bed (Velz, 1948), and the logarithm of the proportion of remoeable matter in the samples was plotted as ordinate against depth of the filter bed as abscissa. Since a linear relationship resulted from this it proved that removal of organic matter in the filter bed was by a first order reaction. This has indicated (Schulze, 1960) that, below a limiting load, the efficiency of a trickling filter, measured as the fraction of removeable organic matter remaining in the effluent, is constant.

To refine the constants of the first order reaction; corrections have been introduced for temperature (Velz, 1948), hydraulic loading (Schulze, 1960) and for flow-through characteristics (Eden, Brendish & Harvey, 1966). However, this has not resulted in precise prediction of the performance of trickling filters either experimentally or in practice. For instance Gloyna et. al.(1952) found that the efficiency of purification of domestic sewage decreased with increased organic loadings.

In the present study, purification of dilutions of piggery wastes (Fig. 11) to less than 1,500 mg/l COD was constant (i.e. in agreement with the theory), but the efficiency of purification of domestic sewage increased with increased organic loadings of the waste (Fig. 11). The methods used to measure the strengths of the wastes and effluents may explain why these discrepancies should occur. BOD was used by Gloyna et al. (1952) and COD was used in the present study, and neither can be regarded as a suitable index for testing the theoretical consideration (Velz, 1948). An increase in the organic loading, measured by either of thesemethods, does not take into account whether the increase lies in the biodegradable or the recalcitrant fractions of the waste.

In addition to these difficulties, constants derived from the theory (Velz, 1948) may vary for different filter beds. For instance, when six large pilot-scale filter beds were operated under identical conditions for two years (Bruce, 1969) wide variations were found in the quality of the effluents and the accumulation of film. Considerations of trickling filters as applications of continuous microbial culture may be of value in resolving some of these problems, as they have been for activated sludge (Downing & Wheatland, 1962).

Systems, such as trickling filters, in which the composition of the medium (waste) changes along the direction of flow according to a rate gradient are classed as heterocontinuous culture techniques (Ricica, 1958). Other industrial applications of this technique have included penicillin and alcohol production by microorganisms. Penicillin production was investigated (Clifton, 1943) by allowing the medium to flow through a modified vinegar vat in which the mould grew as a film on wood shavings, and by allowing the mould to grow on the external surface of cellophane tubing through which the medium flowed (Lewis & Lucas, 1945). Microbial films have been developed in long horizontal glass tubes through which medium flows, and used for the cultivation of Bacterium aerogenes and for production of alocohol and penicillin by brewers' yeast and Penicillium notatum respectively (Moyer, 1929). However none of these methods has had successful industrial application and theoretical considerations on their dynamics are scarce compared with the extensive literature on the homocontinuous culture methods in which the medium composition is the same throughout the culture space.

The mathematical theory of continuous culture can be derived from two basic features of bacterial growth (Herbert, Elsworth & Telling, 1956). Firstly, there is a simple relationship between the growth rate of bacteria and the utilization of a substrate (Monod, 1942). In its simplest form, in growth media containing a single organic substrate, the growth rate is a constant fraction of a substrate utilization rate :

$$\frac{\text{Weight of bacteria formed}}{\text{Weight of substrate used}} = Y,$$

where Y is known as the yield constant. Secondly, the specific growth rate of bacteria is a function of the concentration of an essential substrate when this is low, but above a saturation value the growth rate is limited. All continuous culture systems consist essentially of some form of reactor into which reactants flow at a steady rate and from which products emerge. From the two features above a mathematical theory can be derived which allows quantitative prediction of continuous culture at different flow rates, medium concentrations etc. The most important prediction of the theory is that a continuoue culture is an inherently stable system. As long as the flow rate and composition of the incoming medium remains unaltered, the culture adjusts itself to a steady state in which the concentration of microorganisms and nutrients remain constant.

Application of the theory of continuous culture to the operation of trickling filters is extremely difficult and attempts to explain why the same relationships which describe growth in pure culture and in mixed cultures have been generally unsuccessful (Pipes, 1966). Very little information is available on the nature and concentration of individual compounds in wastes, and the parameters for measuring pollution give only limited or no information on the properties of a waste for biodegradation. The activated sludge process has been investigated (Gaudy & Gaudy, 1966) from the quantitative description of growth of natural microbial populations and of the concomitant concentration changes of individual organic compounds which would be expected to be present in wastes. It is tempting to suggest that a more extended and a more efficient application of trickling filters would result from such an approach to their fundamental considerations.

THE BACTERIAL FLORA OF AEROBIC WASTE TREATMENT

Trickling filters and the activated sludge process provide environments which are selective for aerobic organisms capable of the rapid utilization of the major non-recalcitrant substrates in the wastes. The dominant organisms are those which possess the greatest activity in an environment and thus control the growth of other organisms (Brock, 1966). There are normally other organisms (the associated organisms) which are dependant for their development on the activities of the dominant group. The dominant and associated groups of organisms together, comprise the indigenous flora. However it is possible to distinguish a third group, the transients, which are carried through the system in the waste and make little or no contribution to purification.

The treatment plant provides a favourable environment for selecting and maintaining the bacterial flora responsible for purification. In trickling filters the indigenous bacteria adhere to a support medium upon which they develop as a film. In the activated sludge process they are attached to inert particles where they grow in masses embedded in capsular material. The processes can be regarded as containing the same essential features (Renn, 1956), and it has been demonstrated in the laboratory (Butterfield & Wattie, 1941) that bacteria isolated from trickling filters or activated sludge may be used interchangeably for each process. Although the present study has been concerned with trickling filters, investigations of their bacterial flora have been rare because of the difficulty in obtaining representative samples. The activated sludge process has lent itself much more easily to experiments in the laboratory. As a result, literature on the activated sludge process comprises the major part of the information on the bacterial flora of aerobic waste treatment processes.

The role of bacteria in trickling filters was first recognised by Johnson (1914), and in activated sludge by Russel & Bartow (1916). The first investigators of activated sludge (Russel & Bartow, 1916) were of the opinion that aerobic spore formers were the predominant bacteria, whilst others (Harris, Anderson & Cockburn, 1927) stressed the importance of members of the Enterobacteriaceae. In the light of subsequent work (Allen, 1944) it is tempting to suggest that spore forming bacteria and organisms of intestinal origin are transient and contribute little to purification of wastes. The filamentous sheathed bacteria, usually identified with Sphaerotilus have been noted frequently in trickling filters (Johnson, 1914; Holtje, 1943) and in activated sludge (Buswell & Long, 1923; Buswell, 1931) where they have been associated with failure of the sludge to settle (Smit, 1934; Ingols & Heukelekian, 1939). Buswell & Long (1923), as a result of microscopical examination, concluded that activated sludge was composed of jelly-like (zoogloeal) masses in which bacteria were embedded. Bacteria capable of forming zoogloea have been isolated from trickling filters (Johnson, 1914 ; Butterfield & Wattie, 1941) and activated sludge (Taylor 1930; Butterfield, 1935; Winogradsky, 1937). Many investigators have identified them with Zoogloea ramigera (Gram-negative rod-shaped bacteria contained in a gelatinous matrix) and they have contributed to the view that the zoogloeal condition and the property of floc formation in activated sludge was peculiar to this species (Butterfield, Ruchhoft &McNamee, 1937; Butterfield & Wattie, 1941, Wattie, 1943).

However, subsequent studies have shown that zoogloeal matrices are common to other organisms which have been isolated from waste treatment processes (McKinney & Horwood, 1952; McKinney & Weichlein, 1953; James, 1964) and which have been identified with other genera. Neither the original type strains of Z. ramigera nor those isolated by others (Butterfield et. al., 1937; Butterfield & Wattie, 1941; Wattie, 1943) are extant. Therefore there is insufficient evidence to decide whether Z. ramigera, as identified by the early workers, is a taxospecies/genospecies (Jones & Sneath, 1970) or merely a nomenspecies for organisms which are capable of forming zoogloeal matrices in sewage (Eckenfelder & O'Connor, 1961; Hawkes, 1963).

The jelly-like matrix has provided an impediment to investigations concerning the enumeration and identification of bacteria from waste treatment processes. By overcoming this problem through homogenizing activated sludge before bacteriological examination, Allen (1944) recovered 10 to 100 times more bacteria and contributed to a better understanding of the microbiology of these processes. Breaking up the floc allowed isolation of bacteria from its interior and their distinction from the smaller number of bacteria in the surrounding liquid. Recent work (Williams, Stafford, Callely & Hughes, 1970) has demonstrated the superiority of ultrasonic techniques over older methods for the disruption of flocs. The media used for the isolation of bacteria may also have restricted the number and range of organisms recovered from waste treatment processes. Jasewicz & Porges (1956) used a milk agar, Rogovskaya & Lazareva (1959) used a meat-extract agar, Dias & Bhat (1964) identified organisms which survived transfer to a proteose-peptone

yeast extract broth, and McKinney & Weichlein (1953) and James (1964) used nutrient agar. All these investigators isolated only bacteria which grow well in media containing high concentrations of protein or protein hydrolysis products. Yet it has frequently been shown (Topping, 1937; Keddie, Leask & Grainger, 1966) that media containing low concentrations of nutrients are most suitable for the isolation of a wide range of bacteria from natural environments such as soil.

The majority of organisms isolated by Allen (1944)were Gram-negative rods which failed to produce acid from carbohydrates and were identified with Achromobacter, Flavobacterium or Pseudomonas as defined in Bergey's Manual of Determinative Bacteriology (Bergey et al., 1934). In subsequent studies (McKinney & Weichlein, 1953; Jasewicz & Porges, 1956; Rogovskaya & Lazareva, 1959; Dias & Bhat, 1964; James, 1964; Van Gils, 1964; Adamse, 1966) of the bacterial flora of waste treatment processes (Table 8), these genera and Alcaligenes, Comamonas, Zoogloea and members of the Corynebacteriaceae have been described as dominant, and a great many other genera have been noted also. However in a consideration of the frequency with which organisms have been noted, four groups can be recognised.

The first (Table8) includes organisms which have been described in the majority of studies and which have normally been recovered in large numbers. They can be regarded as the dominant organisms and they have been identified with Achromobacter, Alcaligenes, Flavobacterium and Pseudomonas. In contrast, the organisms of Group IV

(Table 8) have been recovered infrequently and in low numbers, these can be regarded as transients which contribute little to the changes occuring in the ecosystem. Organisms in Group II (*Micrococcus* and *Bacillus*) are common but have never been isolated in large numbers, whereas Group III organisms (*Comamonas*, *Zoogloea* and the coryneforms) have been isolated infrequently but usually in large numbers. Some of the organisms in Groups II and III may belong with the dominant or transient organisms whereas others may comprise an associated group dependant for their development upon the dominant organisms.

In the present study the bacteria which were considered to comprise the dominant flora of films developing on experimental trickling filters were identified with *Acinetobacter*, or were characterised as yellow-pigmented non-motile Gram-negative rods. Dominant organisms were not identified with any of the four genera (*Achromobacter*, *Alcaligenes*, *Flavobacterium* and *Pseudomonas*) which the literature (Table 8) would suggest are dominant in waste treatment processes. However, identification of a bacterial isolate is achieved by comparison with named types described in treatises on determinative bacteriology, and the current definitions of named types tend in many instances to differ from those of earlier usage.

Acinetobacter are short rod - or coccoid rod-shaped non-pigmented bacteria which occur mainly in pairs or short chains. With Gram's stain they are negative but have a tendency to retain the crystal violet (Henriksen, 1952; Thonley, 1967); they are oxidase-negative and non-motile. They do not ferment carbohydrates but oxidise them to acid products by an obligately aerobic process or leave them apparently unchanged. Although the name *Acinetobacter* was first validly published by Brisou & Prévot

(1954) their definition was not included in the 7th edition of Bergeys' Manual of Determinative Bacteriology (Breed et al., 1957) In this and a previous edition (Breed, Murray & Hitchens, 1948), on whose definitions identification of organisms listed in Table 8 were based, Gram-negative rod-shaped non-pigmented bacteria, either motile by peritrichous flagella or non-motile, were defined as Achromobacter or Alcaligenes. However, computer analyses (De Ley, Bain & Shewan, 1967; Thornley, 1967) of morphological, physiological and biochemical data, and measurement of deoxyribonucleic acid (DNA) base composition, have shown that non-motile strains formerly identified with Achromobacter or Alcaligenes are distinct from those having peritrichous flagella. Non-motile strains are considered to form a homogeneous group to which the name Acinetobacter has been given (Thornley, 1967; Baumann, Doudoroff & Stanier, 1968 b), although this may not prove to be the most appropriate choice of name (see page 101). It is tempting to suggest that isolates from waste treatment processes previously identified with Achromobacter or Alcaligenes might be now correctly identified with Acinetobacter. The work of Robinson, Baxter & Saxon (1970) which showed that Acinetobacter was the predominant bacteria in activated sludge from a Pasveer oxidation ditch treating piggery wastes, has corroborated the findings of the present study. However, from the literature it is not possible to decide whether isolates identified with Achromobacter and Alcaligenes by previous workers (Table 8) were motile.

The other dominant bacteria in the present study were yellow-pigmented non-motile Gram-negative rods. According to the 7th edition of Bergeys' Manual of Determinative Bacteriology (Breed et al., 1957) organisms having these properties can be identified with Flavobacterium. The original definition of this genus (Bergey et al.,

1923) accommodated virtually any yellow-pigmented rod, and even though subsequent revision has limited it to Gram-negative strains (Breed et al., 1957) a large number of Gram-positive types have been included in the genus (Dorey, 1959). At the present time adequate means of defining Flavobacterium are not available. In an effort to render this a useful taxon Weeks (1955) designated a neotype species Flavobacterium aquatile strain Taylor (ATCC 1947) but this has been shown (Dworkin 1966) to be similar to, and impossible to distinguish from, the cytophagas and sporocytophagas. As a result, the recent approach to the identification of yellow-pigmented rods (Hendrie, Mitchell & Shewan, 1968) has centred on their possible relationship to better defined genera. It has not been possible to find a better identity for the yellow-pigmented rods reported in the literature on waste treatment processes and which were identified with Flavobacterium. In the present study none of the yellow-pigmented rods had flagella. Hendrie et al. (1968) suggested Cytophaga, Sporocytophaga and the fruiting myxobacteria as possibly better-defined groups for yellow-pigmented rods without flagella. The principal characteristics of these organisms, flexous cell walls and gliding motility on solid substrata, were not observed in the isolates obtained in this study but there are many difficulties (Lautrop, 1965) in unequivocally demonstrating such features. However, since organisms which have neither flexous cells nor gliding motility have been identified with Cytophaga as a result of theirDNA base composition (Marmur, Falkow & Mandel, 1963), this genus cannot be disregarded as a possible location for the yellow-

pigmented Gram-negative rods which were isolated in the present study. It is considered (Weeks, 1969) that the identification of such organisms awaits detailed comparative studies with cytophagas before definite conclusions may be reached.

Although organisms identified with Pseudomonas were not isolated in great numbers in the present study, they have featured prominently in other investigations (McKinney & Weichlein, 1953; Rogovskaya & Lazareva, 1959; James, 1964; Adamse, 1966). Stanier, Palleroni & Doudoroff(1966) reaffirmed the view of Kluyver & Van Niel (1936) that Pseudomonas should be defined as Gram-negative unicellular rod-shaped bacteria motile by one or more polar flagella, their energy yielding mechanism being by respiration using molecular oxygen as the terminal oxidant. Within this definition are included a large and fairly diverse array of bacteria which are most commonly classified in other genera: Xanthomonas, Methanomonas, Hydrogenomonas, Comamonas, Vibrio sensu lato, Acetomonas (Gluconobacter), and possibly also Alginomonas, Cellulomonas and Cellvibrio (Stanier, et al., 1966). Comamonas and Zoogloea, which have been isolated infrequently (Table 8) from waste treatment processes but usually in large numbers may be better identified with Pseudomonas. Dias & Bhat (1964) have been the only workers to isolate Comamonas in large numbers from waste treatment processes. Identification was achieved by comparison with the definition of Comamonas as rod-shaped bacteria which have polar flagella and do not produce fluorescent pigments nor use carbohydrates as sole carbon sources (Park, 1962). There is evidence, however, that lophotrichous

flagella are the unique feature of this genus (Hugh, 1962) and it seems that the isolates of Dias & Bhat (1964) may be better identified with Pseudomonas. In the work of Dias & Bhat (1964) it was only the absence of a gelatinous matrix which distinguished their isolates of Comamonas from those which they identified with Zoogloea. Cohn (1854) defined the genus Zoogloea and one species, Zoogloea termo. The Latin description has been translated (Zvirbulis & Hatt, 1967) thus : Cells very small, rod-shaped colourless; hyaline jelly in spherical mucilaginous lumps; resembling a cluster of grapes, at first assembled as in a membrance, later individually separated, moving to and fro in the water. Fourteen years laterItzigsohn (1868) described Z.ramigera as Gram-negative rod-shaped bacteria contained in a gelatinous matrix. Since the formation of gelatinous masses (flocs) is a distinctive feature of the activated sludge process there has been a tendency (Crabtree & McCoy, 1967) to attribute the capability of floc formation to the one organism, Z. ramigera. However the sole criterion which distinguishes Z. ramigera from non-fluorescent pseudomonads is the production of polymerised capsular material, the zoogloeal matrix (Friedman & Dugan, 1968; Friedman et al., 1968) and this has been shown to be a feature of several other organisms (Butcher, 1932; James, 1964) when grown under certain conditions. Since the gelatinous matrix is neither a constant nor exlusive property, organisms currently identified with Zoogloea may be considered to conform to the definition of Pseudomonas (Stanier, et al., 1966)

Another group of organisms which have featured in the literature on waste treatment processes in large numbers are bacteria having coryneform morphology (Adamse, 1966). In the present study they were

isolated from films developing on experimental trickling filters irrigated with domestic sewage(Fig. 9) but were not considered to be dominant. Coryneform bacteria are irregular club - or wedge - shaped cells having a positive to variable Gram-reaction, sometimes irregularly staining, and a "snapping" form of cell division which results in an angular arrangement of cells (Jensen, 1952). The majority of those which have been isolated from waste treatment processes (Van Gils, 1964; Adamse, 1966) have been identified with Arthrobacter. In this genus a growth cycle, common to all coryneform bacteria (Veldkamp, 1970), is particularly evident, irregular rods in young cultures break down to coccoid forms in older cultures (Keddie et al., 1966). It may be that coryneforms are more important in waste treatment than is apparent from the limited number of occasions on which they have been isolated (Table 8). Arthrobacters make a considerable contribution to the microflora of soil (Topping, 1937; Lochhead & Burton, 1953; Rouatt & Katznelson, 1961; Sperper & Rovira, 1959) but absence of growth factors (Lochhead & Burton, 1953) and/or a high nutrient concentration (Keddie et al., 1966) may suppress their development in laboratory media. If unsuitable media have been used for their isolation (see page 88) and/or the coccoid forms of older cultures have been mistakenly identified with micrococci, it is possible that the coryneforms might constitute a dominant group whose contribution to waste treatment has been overlooked.

Although it may be expected that variation in the composition of wastes will be reflected by variation in the bacterial flora involved in purification, the organisms associated with waste treatment processes belong to only a few main groups. These are Pseudomonas

(aerobic pseudomonads), achromobacters (organisms which have been identified with Achromobacter or Alcaligenes but may possibly be correctly identified with Acinetobacter), flavobacteria (yellow-pigmented organisms which have been indentified with Flavobacterium but may be correctly identified with other genera), and possibly coryneforms. Table 9 has been constructed to show the incidence with which the pseudomonads, achromobacters and flavobacteria have been recovered in large numbers from waste treatment processes. It is evident in all but two of the investigations (James, 1964; Adamse, 1966) that when pseudomonads were present in large numbers, achromobacters and flavobacteria were absent, and vice versa. It is tempting to suggest that two types of microbial associations occur in waste treatment processes, one dominated by pseudomonads, and the other by achromobacters and flavobacteria. The two exceptions were unusual in that one (James, 1964) was from a study of trickling filters, and the other (Adamse, 1966) from a Pasveer oxidation ditch. These are situations where two types of flora could have been located separately and not in direct competition with each other. It has been suggested (Adamse, 1966) that purification of proteinaceous wastes is dominated by achromobacters and flavobacteria whilst carbohydrate wastes favour pseudomonads, but it is not considered that the composition of the wastes in Table 8 corroborate this view. In the present study the microflora of films developed on experimental trickling filters irrigated with domestic sewage was of the achromobacter/flavobacteria type. It would seem probably that the presence of both organisms was necessary for effective purification. It is tempting to suggest that the absence of flavobacteria from the microflora was the cause of less efficient purification when the film was irrigated with piggery wastes (Fig. 11).

Taxonomy of Gram-negative, non-motile diplobacilli (Acinetobacter)

In the present study one of the dominant organisms isolated from films developing in experimental trickling filters was identified with Acinetobacter, and it has been suggested (see page 90) that this might be a better generic location for isolates identified with Achromobacter or Alcaligenes in other investigations. The distinguishing characteristics of organisms which were identified with Acinetobacter are that they were short rods or cocci occurring mainly in pairs or short chains but with longer rods, filaments and swollen forms. They were Gram-negative, but had a tendency to retain the violet colouration, oxidase-negative and without flagella. They did not ferment carobhydrates but either dissimilated them to acid products or left them apparently unchanged.

Organisms with these characteristics have become prominent in current literature because of the wide range of habitats from which they have been isolated, and because of the considerable difficulties encountered in their classification. They have been isolated from soil (Lemoigne, Girard & Jacobelli, 1952; Holding, 1960), water (Baumann, 1968), activated sludge (Robinson et al., 1970), meat (Gardner, 1965), poultry (Thornley, 1960; Thornley, Ingram & Barnes, 1960), fish (Shewan, Hobbs & Hodgkiss, 1960 a, b) and clinical sources (Rosebury, 1962). Among the genera to which they have been assigned are Micrococcus, Neisseria, Pseudomonas, Diplococcus, Alcaligenes, Bacterium, Achromobacter, Cytophaga, Moraxella, Herellea, Mima and Acinetobacter.

The confusion in the taxonomy of these bacteria may be resolved in two stages : 1) acknowledging that they are a unique group of species or biotypes; and 2) assigning to them a generic location and type species. It is widely accepted (Thornley,

1967; Baumann et al, 1968 b) that they constitute a taxospecies, so it remains to discuss the generic locations into which they have been placed. Some generic names which have been applied to these organisms are obviously unsuitable, for instance Micrococcus and Diplococcus are Gram-positive, Neisseria is coccoid, and Pseudomonas and Alcaligenes have flagella. However their relationships to the genera Bacterium, Achromobacter, Cytophaga, Moraxella, Herellea, Mima and Acinetobacter merit discussion.

Bacterium has been recognised as a nomen genericum rejiciendum (Judicial Commission, 1954), but was once reserved for organisms which could not be assigned to any of the recognised genera. Schaub & Hauber (1948) isolated a morphologically and biochemically homogeneous group of Gram-negative bacilli from human sources which, because their taxonomic position was not clear, they placed in Bacterium. These organisms were short, coccoid, non-motile bacilli resembling Neisseria in Gram-stained preparations. Their failure to reduce nitrates differentiated them from the majority of Gram-negative bacilli, and the specific epithet anitratum was selected because it expresses this characterstic. Bacterium anitratum (Schaub & Hauber, 1948) has been shown to belong with the group of bacteria under discussion (Thornley, 1969; Baumann et al., 1968 a, b). Since Bacterium has become nomen genericum rejiciendum (Judicial Commission, 1954) there have been proposals to transfer B. anitratum to several other genera; Achromobacter (Brisou & Moricheau-Beauchant, 1952; Stenzel & Mannheim, 1963), Cytophaga (Lautrop, 1961), Moraxella (Piéchaud, Piéchaud & Second, 1951), Herellea and Mima (Ewing, 1949), and Acinetobacter (Brisou & Prévot, 1954).

Achromobacter is a group of obviously unrelated Gram-negative rod-shaped bacteria which have been defined (Breed et al., 1957) as either motile by means of peritrichous flagella or non-motile. However it has been demonstrated (De Ley et al., 1967; Thornley, 1967) that the motile strains are distinct from those having peritrichous flagella. The description of the type species Achromobacter liquefaciens (Bergey et al., 1923) as having peritrichous flagella suggests that non-motile strains should be excluded from this genus. However identification of isolates with this genus has been made particularly difficult because inadequate early descriptions of its type species have made it difficult to re-isolate an acceptable type culture (Breed et al., 1957).

Cytophaga is principally characterised by having flexous cell walls and gliding motility on solid substrata (Stanier, 1942). A type of movement which was shown by strains of B. anitratum and Moraxella lwoffi was identified with gliding, and prompted Lautrop (1961) to propose their transfer to Cytophaga. Both B. anitratum and M.lwoffi have been included in the taxospecies under consideration (Baumann et al., 1968 b). However these two organisms differ substantially in their phenetic characteristics (Seeliger, Schubert & Schlieber, 1968) from Cytophaga. Furthermore, the unsuitability of Cytophaga as a generic location was undermined when it was noted (Schlieber, 1964; Lautrop, 1965) that the movement of B. anitratum was a twitching, jerky motility which was not found in the Cytophaga strains used for comparison.

Lwoff (1939) questioned the inclusion of Gram-negative, non-motile diplobacilli in Haemophilus, and proposed Moraxella for these organisms

which had been previously identified with Haemophilus lacunata. Recent evidence suggests that oxidase-negative penicillin resistant organisms are distinct from Moraxella. The oxidase reaction has been shown (Baumann et al., 1968 a) to be correlated with the presence or absence of cytochrome c, and penicillin resistance is not attributable to production of penicillinase. DNA base compositions (Sebald & Véron, 1963; Baumann, et al., 1968 b) of oxidase-negative stains are around 39-41 moles % guanine plus cytosime (GC), whereas the characteristic value for all the moraxellas is around 44 moles % GC (Catlin & Cunningham, 1964 a; Henriksen & Bøvre, 1968). Transformation has been reported for both oxidase-positive stains (Bøvre, 1964; Catlin & Cunningham, 1964 b) and oxidase-negative stains (Juni & Janik, 1969). However, although inter-species transformation has been observed within the oxidase-positive moraxellas, DNA from oxidase-negative strains is not known to be capable of significantly transforming oxidase-positive strains. Therefore it has been widely advocated (Henriksen, 1952, Baumann et al., 1968 a, b; Henriksen & Bøvre, 1967, 1968; Nicoli, Bideau, Vandekerkove & Faucon, 1970) that Moraxella is an unsuitable generic location for oxidase-negative bacteria.

A tribe Mimeae was proposed by De Bord (1942) for bacteria which could not be identified with existing genera. The three genera of this tribe were separated on the basis of their action on carbohydrates; Mima, carbohydrates not attacked; Herellea, acid produced from carbohydrates; and Colloides, acid and gas produced from carbohydrates. The latter has subsequently been shown (Henriksen, 1952) to be related to Escherichia freundii as defined in Bergeys' Manual of Determinative Bacteriology (Breed et al., 1948). On the basis of morphology and action on carbohydrates, Mima and/or Herellea are possible generic names for the Gram-negative,

non-motile, oxidase-negative diplobacilli. However, neither were well defined and several investigators (Henriksen, 1952, 1963; Rosebury, 1962; Pickett & Manclark, 1965; Baumann et al., 1968 a) have recommendedthat they should be abandoned. Mima was defined as either oxidase-positive or negative and excluded strains producing acid from carbohydrates, and Herellea was defined as either motile or non-motile and as attacking mannitol and dulcitol, features which have not been demonstrates in detailed studies of Gram-negative, non-motile, oxidase-negative diplobacilli (Thornley, 1967, Baumann et al., 1968 b).

Acinetobacter was defined (Brisou & Prévot, 1954) so that non-motile organisms could be excluded from Achromobacter and the inclusion of the Gram-negative, non-motile, oxidase-negative diplobacilli in Acinetobacter has been widely advocated (Brisou, 1957; Steel & Cowan, 1964; Thornley, 1967; Henriksen & Bøvre, 1968; Baumann et al., 1968 b). However, Acinetobacter was not well defined (Seeliger et al., 1968). It was placed in the Achromobacteraceae, a family which is deemed to consist of organisms having peritrichous flagella. The type species was designated as Acinetobacter (Bacterium) anitratum by Brisou (1957) and as Acinetobacter stenohalis by Prévot (1961). The earlier proposal (Brisou, 1957) has been withdrawn (Seeliger et al., 1968) and the obligate halophile Ac. stenohalis has become the legitimate type species. Therefore, since the group of organisms under discussion are neither motile nor typically halophilic, Acinetobacter does not constitute a suitable generic location. In the present study Gram-negative, non-motile, oxidase-negative diplobacilli have for convenience been identified with Acinetobacter merely because this genus has enjoyed a wide acceptance.

At present there is no widely accepted generic location for these organisms. However, their principal biotype has been shown (Thornley, 1967; Baumann *et al.*, 1968 *b*) to be organisms which had previously been identified with *B. anitratum.* A new genus, *Lingelsheimia*, has recently been proposed (Seeliger *et al.*, 1968) for organisms identified with *B. anitratum*. It is considered that *Lingelsheimia* may be the most suitable genus for organisms which have been previously identified with non-motile achromobacters, oxidase-negative moraxellas, non-motile and oxidase-negative *Mimeae*, strains of *Acinetobacter* without flagella, and *B. anitratum*.

It has been suggested (Ryter & Piéchaud, 1963; Seeliger *et al.*, 1968) because of differences in cell wall structure to other eubacteria that *Moraxella* and *Lingelsheimia* be placed in a new family, the *Moraxellaceae*. However, the validity of this proposal is in doubt because Thornley & Glauert(1968) have shown that there is little difference between cell walls of *Acinetobacter* (*Lingelsheimia*) and other Gram-negative bacteria. Alternatively, on the basis of phenetic characteristics and genetic evidence from DNA base composition and transformation (Henriksen & Bøvre, 1968) it has been proposed (Henriksen, 1952) that *Moraxella* belongs in the *Neisseriaceae*. Transformation experiments (Catlin & Cunningham 1964 *b*; Bøvre, 1967) do not indicate a close relationship for either *Moraxella* or *Neisseria* with *Lingelsheimia* (*Acinetobacter*). The genetic evidence is, at present, too sparse to be conclusive. It has been demonstrated (Adams, Quadling, Yagachi & Tonabene, 1970) that the chemical composition of the cell wall lipopolysaccharide of *Lingelsheimia*, *Moraxella* and *Neisseria catarrhalis* is similar, and differs from that of typical neisserias such as *Neisseria gonorrhoea*. However, until further evidence is forthcoming the familial relationships of these organisms must remain in doubt.

BACTERIAL ECOLOGY OF TRICKLING FILTERS

In ecology, the study of the relationships of organisms to their environments, the concept of an ecosystem provides a framework within which studies can be meaningfully conducted. An ecosystem is usually defined (Brock, 1966; Alexander, 1971) as the total of the interacting factors (including both biological and abiotic components) in a limited environment. In waste treatment processes the physical and chemical parameters select the microorganisms which can develop populations or associations and the changes they can cause in the waste. Operational procedures create conditions whereby microorganisms which are concerned in the decomposition of organic materials in the waste are encouraged to grow at the expense of others. Trickling filters provide a habitat (the place where a community lives) for organisms which can develop as a film on the surfaces of a support medium over which the waste flows, and a niche (the role that organisms play in the ecosystem) for those which are able to oxidise the organic materials in the waste. It is the purpose of this section to set out what is known of the constitution and activities of the microorganisms which occupy this ecosystem in order to attempt to explain the action of trickling filters.

The purifying action of trickling filters is dependant upon the activity of microorganisms which colonise the surfaces of the support medium. There is evidence(Zobell & Allen, 1934) that bacteria attach themselves to solid surfaces by cementing substances rather than in any passive way. This substance has been considered (Zobell, 1943) to be neither lipid nor calcareous, and recent work (Jones, Roth & Saunders, 1969) suggests that it is a matrix of poorly soluble polysaccharide-like material. It is tempting to suggest that it is an

extension of the bacterial cell surface which is responsible for its attachment. Typically, although it has been claimed (Zobell, 1943) that there is no relationship between the Gram-reaction of pure cultures of bacteria and their attachment propensities, it seems that bacteria which develops as films are mainly Gram-negative (Henrici, 1933; James, 1964). It is interesting that a wrinkled pattern, derived from particular organisation of protein material, has been noted on the surface of Gram-negative bacteria (Thornley & Glauert, 1968) in contrast to the smooth surface seen in Gram-positive organisms (Zwillenberg, 1964). The greater physical complexity of the walls of Gram-negative bacteria (Salton, 1961) over those of Gram-positive bacteria may contribute to their adhesive properties. In addition, thread-like surface appendages (fimbriae) have been seen on the surface of many bacteria (Duguid, Smith, Dempster & Edmunds, 1955) notably Acinetobacter (Thornley, & Glauert, 1968). In certain bacteria the fimbriae have been shown to be responsible for adhesion (Duguid & Gillies, 1957), and it is possible that these structures may be widespread in bacteria which attach themselves to solid surfaces.

It is considered that the characteristic features of both trickling filters and the activated sludge process, films and flocs, have arisen because both processes were originally developed for the purification of wastes containing only low concentrations of nutrients, i.e. domestic sewage.Zobell & Grant (1943) showed that bacterial activity is proportional to the concentration of the nutrients when the latter is low. It follows that in very dilute media any factor which tends to concentrate the organic matter would promote bacterial activity. Surfaces are thought to concentrate nutrients thus enabling bacteria to develop on substrates which otherwise would be too dilute. It has been noted that storage of waters, surface, underground, sea and sewage, removed from their

natural environments results in an initial increase in bacterial numbers (Heukelekian & Heller, 1940) and that the greatest increases are obtained in small vessels (Zobell & Anderson, 1936). This was attributed to the contact of the water with a relatively larger surface area in the small receptacles. The work of Heukelekian & Heller (1940) with pure cultures of Escherichia coli corroborated this view. It was found that introduction of glass beads into the culture vessel to increase the surface/volume ratio reduced the limiting nutrient concentration for growth. Inasmuch as this effect of surfaces upon bacterial activity can only be demonstrated in very dilute media it was suggested (Zobell, 1937) that it is the result of nutrients being concentrated on the solid surfaces. It is generally believed that large organic molecules or particulate matter are hydrolyzed by exoenzymes before absorption and assimilation by bacteria. In dilute media these exoenzymes and their hydrolyzates may tend to diffuse away from the cells responsible for their formation thus making it difficult for the bacteria to obtain enough nutrients for their organic requirements. Should surfaces retard the diffusion of exoenzymes and their hydrolyzates, very weak substrate concentrations would be selective for organisms which attach themselves to surfaces. The film which develops in trickling filters would appear to be particularly suited to the removal and oxidation of soluble impurities from wastes of low concentration. In activated sludge the predominant bacteria attach themselves to inert particles where they grow embedded in masses of capsular material (flocs). Substrates are concentrated at the surfaces of the flocs in order to become available to the organisms. Therefore the operational procedures which have been determined for efficient purification by trickling filters and the activated sludge process are the result of selective pressures exerted by wastes (e.g. domestic sewage) containing only low concentrations of nutrients.

However, highly concentrated wastes, such as those from intensively housed pigs, will not impose the same selective pressures because further concentration of the nutrients is unnecessary, Heukelekian & Heller (1940) found that films irrigated with highly concentrated wastes tended to be watery and slough off easily, and in the present study irrigation with piggery wastes did not initiate film accretion on trickling filters. It is possible, therefore, that the ecological basis of aerobic waste treatment processes may restrict their application to very dilute organic wastes.

Many, but not all, bacteria are capable of attaching themselves to solid surfaces, for instance Zobell & Allen (1934) found that only 24 out of 73 morphologically or biochemically differing cultures from sea water became attached to glass slides in the laboratory. Filamentous types have frequently been noted in films (Henrici, 1933; Zobell & Allen, 1934; Heukelekian & Crosby 1956; Dondero, 1961) but have never been considered to comprise a majority of the types of bacteria. Typically bacteria which attach themselves to solid surfaces and develop as films are encupsulated Gram-negative rods which do not ferment carbohydrates (Henrici, 1933). Zobell & Allen (1934) identified at least 60% of the total number of bacteria in films developing on slides immersed in sea water with Gram-negative cocco-bacilli and described these species, Achromobacter marinoglutinosus, Achr. membranoformis, and Flavobacterium amocontactus. In Bergeys' Manual of Determinative Bacteriology (Breed et al., 1957) the two achromobacters are now designated Pseudomonas, and Flavobacterium amocontactus, Agarbacterium amocontactus. In the present study of films the principal bacteria were Gram-negative rods which did not ferment carbohydrates, and were either identified with Acinetobacter or characterised by a yellow-pigmentation and absence of flagella.

The physical and chemical environment of trickling filters is selective for film-forming organisms which can rapidly utilize the major and most easily biodegradable substrates in the waste. Since the process is inoculated continuously with organisms from the waste and the air there must be competition between various bacteria for the same resources. In the present study a film from experimental trickling filters (Gloyna et al., 1952) was examined after 3 days irrigation with domestic sewage, and its bacterial flora was identified with 8 different genera or groups, each present in about the same proportion (Fig.9). After 3 weeks irrigation the proportions of the population represented by two of these groups (Acinetobacter and the Gram-negative yellow-pigmented rods) had increased, one (the coryneforms) remained at about the same level, and the others had decreased.

Various workers, notably Gause (1935), have demonstrated that two species using the same resources cannot coexist indefinitely in the same ecosystem. This has been termed the competitive exclusion principle, and it has been defined (Hardin, 1960) as : (1) if two non-breeding populations occupy the same niche, and (2) if they are sympatric (having the same, or overlapping, habitats), and (3) if A multiplies faster than B, then ultimately A will replace B. Therefore although many varieties of bacteria can occupy a habitat such as that presented by trickling filters only those which occupy the niche or niches most efficiently will become dominant. When these have emerged and efficient waste purification has been attained, the film may be said to have matured. In the present study since two organisms were successful competitors in the ecosystem presented by experimental trickling filters irrigated with domestic sewage, it follows that these organisms (Acinetobacter and the Gram-negative yellow-pigmented rods) do not occupy the same niche. It has been

considered previously (see page 95) that these two types of bacteria form an association which is responsible for waste purification in trickling filters, and that there is an alternative type of flora dominated by pseudomonads. However it remains to resolve the nature of their association and their roles in aerobic waste purification.

In trickling filters operational procedures and the nature of the waste determine which bacteria become dominant. However information on the kinds and concentrations of individual compounds in wastes, particularly sewage, is scarce. Painter & Viney (1959) have published analytical data showing that the principal constituents of domestic sewage were carbohydrates, amino acids (both free and as peptides and proteins), organic acids and esters. A striking feature of both Pseudomonas and Acinetobacter is their ability to utilise a wide range of organic compounds (Stainer, et al., 1966; Baumann, 1968) such as one might expect to be present in wastes. However Acinetobacter is notable for its lack of activity towards carbohydrates (Baumann et al., 1968 b), and in the present study it has been found to be poorly competitive with Pseudomonas except under highly aerobic conditions (see page 31). It is tempting to suggest that under conditions where movement and exchange of gases is restricted and/or in the presence of a waste rich in carbohydrates Pseudomonas might become the dominant organism. Also, since Pseudomonas, for an equivalent reduction of COD, yields a greater amount of cellular material than Acinetobacter (see page 33), it is possible that such a flora is likely to be the result of poor ventilation and to be responsible for clogging and ponding of filter beds.

A distinctive feature of trickling filters (and of activated sludge) is the capsular material within which the organisms are embedded. According to electron microscopy (Saunders, 1966; Jones et al., 1969) this material is also responsible for adhesion and is composed of polysaccharide. Information on the capsular material of Zoogloea ramigera is considered to be applicable to films dominated by pseudomonads. Its fine structure has been shown (Friedman et al., 1968) to be composed of strands of polysaccharide which are slightly soluble in water and whose solubility increased in alkaline solutions. It has been suggested (Friedman et al., 1968) , because of its susceptibility to cellulase and because glucose has been the only component resulting from acid hydrolysis, that the capsular material of Z. ramigera resembles cellulose (i.e. contains 1, 4-β-glycosidic bonds). The capsular materialof Acinetobacter calco-aceticus has also been shown to be a polysaccharide (Taylor & Juni, 1960), but it contains two sugars, L- rhamnose and D-glucose, in a ratio of approximately four to one. Immunochemical analysis (Heidelberger, Das & Juni, 1969) of this polysaccharide has revealed non-reducing end groups of L -rhamnose, and that the L-rhamnose occurs in 1, 3-linked repeating units and that glucose occurs at branch points. Synthesis of the polysaccharide capsule is dependant upon the prescenceof air and an oxidisable substrate(Juni & Heym, 1964). It is, presumably, on the capsular material of films that organic materials from the wastes are concentrated. It has been demonstrated (Friedman & Dugan, 1968) that encapsulated strains of Z.ramigera are capable of concentrating and accumulating twice the quantity of cations (Co^{++}, Cu^{++}, Fe^{+++}, Ni^{++}, Zn^{++}) as non-encapsulated strains. This makes is tempting to suggest that further investigations into the capsular material of films and of organisms which develop as films may lead to a better understanding of the manner in which nutrients

from weak wastes are concentrated.

In the present study, and it is considered that it may be widespread in waste treatment systems (see page 95), the microflora of films developing on experimental trickling filters was dominated by an association between Acinetobacter and a Gram-negative yellow-pigmented rod. When a film which was dominated by this association was irrigated with dilute piggery waste, the efficiency of purification decreased, the film lost its brown pigmentation and Acinetobacter became the dominant organism. Clearly, in waste treatment processes Acinetobacter is benefitting from its association with the yellow-pigmented rod but it is not known whether their interaction is one of commensalism (one member benefits whilst the other is unaffected) or of mutualism (each member benefits from the other). Although Acinetobacter is nutritionally versatile (Baumann et al., 1968 b) and has nuclease and lipolytic activities (Barnes & Melton, 1971) it is not known to be active against polymers. For instance, although organisms identified with Acinetobacter have frequently been isolated from environments which could be expected to be rich in protein e.g. meat (Gardner, 1965) poultry (Thornley, 1960) and fish (Shewan, Hobbs & Hodgkiss, 1960 a, b), Acinetobacter is not known to be proteolytic. However the Gram-negative yellow-pigmented rod investigated in the present study was shown to be capable of liquefying gelatin (Table 6). The principal genus with which this organism might be identified is considered to be Cytophaga, comprising organisms which are notable for their activity in breaking down polymers such as proteins, cellulose, chitin etc. Therefore it is tempting to suggest that the association between Acinetobacter and these organisms derives from a requirement by Acinetobacter for the yellow-pigmented rod to break down polymers into assimilable units. The feature of the piggery waste which was responsible for the elimination of the yellow-pigmented rods from the association in the experimental

trickling filters is not known. It may be that some constituent of the piggery waste was inhibitory. Alternatively, if there were sufficient small molecules in the waste to support the nutritional requirements of Acinetobacter then there would be no necessity for the association. However, mixed culture phenomena are not merely composites of the pure culture behaviour of the organisms present (Bungay & Bungay, 1968) and it is not implicit that Acinetobacter could occupy as efficiently the niche once filled by the association. In fact, when the film was irrigated with piggery waste both purification and adhesion was impaired. It is tempting to suggest that further investigations into the association between Acinetobacter and yellow-pigmented rods may help lead to environmental control of populations which are active in aerobic waste treatment.

PRACTICAL CONSIDERATIONS

PRACTICAL CONSIDERATIONS

In the United Kingdom because there is little or no spare land to bring into cultivation and existing farm land is being lost to urban development, the farmer is having to use the available land more efficiently. Technological progress in the pig industry has resulted in intensive housing (Mosley, 1967,) with increasing attention being given to rearing more pigs per sow each year, breeding the "right" pig, improving the food conversion rate, and designing or adapting buildings to provide an appropriate environment at all stages of the pigs' life. The pattern has been one of substantial increases in the average size of herds (Anon, 1968 b), notably in the major pig producing areas in the East and South-West. This has reduced the area of land required to support a given number of pigs but has increased the volume per acre of wastes produced by the animals. It is generally acceptedthat the most satisfactory means of disposal of farm wastes is to spread them on to the soil, but in intensive operations there may be insufficient land available to meet the needs. Where land is not available the problem of disposal of wastes from intensive pig production units is likely to be severe.

Physical and chemical means of disposal

One of the implications of intensive pig housing is that little or no bedding is used (Soutar & Baxter, 1968), instead the animals are kept on a fully or partly slatted floor beneath which is a dung channel from which the wastes are flushed as a slurry. The fluidity of the slurry considerably simplifies its handling but its high moisture content is likely to make economically impractical the principal physical and chemical methods of disposal (drying and

combustion).

Aerobic biological methods of disposal

Aerobic biological waste disposal methods depend upon the growth of microorganisms which, in the presence of air, convert organic compounds into cellular material, CO_2, H_2O and ammonia. The removal of the microorganisms should, ideally, leave an effluent of a standard acceptable under the Rivers (Prevention of Pollution) Acts, 1951 and 1961 (Anon, 1951, 1961 b). The many different operational procedures which exploit the principles of aerobic biological waste treatment can be classified under three headings according to the means by which aeration is achieved. These are, (i) by exposing a large but tranquil surface to the atmosphere, (ii) by violent agitation, or (iii) by allowing a thin film of waste to pass over a microbial slime.

Aerobic lagoons (oxidation ponds) attempt in a restricted area to produce the purification process which takes place in watercourses. The waste is held in a shallow pond where organic material is aerobically decomposed by bacteria. The products of this activity, along with light energy, permit photosynthesis by algae and the oxygen produced in this process becomes available for bacterial metabolism. Dead algae precipitate as a sludge and the cycle repeats itself. The essential requirement for successful operation of an aerobic lagoon is a sufficiently large surface area and this is dictated by the ambient conditions of light and temperature. Low temperatures retard the activities of the microorganisms and may lead to accumulation of organic material in the lagoon over winter and, unless this is broken down in summer, malodorous anaerobic conditions may result (Pratt et al, 1968). This problem can

be controlled and the quality of the effluent improved by artificial aeration of the lagoon and by this means they have been successfully used in the U.S.A. for the treatment of piggery and poultry wastes (Pratt et al., 1968). However, it has been estimated (Pontin & Baxter, 1968) that in the United Kingdom aerobic lagoons would have to occupy 1 acre per 100 pigs, so it is unlikely that they will prove to be acceptable for large enterprises.

Those waste treatment systems in which aeration is achieved by vigorous agitation are referred to as the activated sludge process. The waste is added to a community of microorganisms which is continuously agitated by injection of air through spargers or by mechanical means at the surface. The modification of the activated sludge process which has been considered for the treatment of piggery wastes is the Pasveer oxidation ditch, a method developed by Pasveer (1959) for economical purification of wastes from villages, small factories etc. Because aeration is in a tank of the simplest possible construction (usually an oval ring-shaped earthen ditch) the oxidation ditch has a low initial cost. A rotor is mounted in the ditch to provide aeration and circulation of the waste around the ditch. Apparently Scheltinga (1966) first used an oxidation ditch for purification of piggery wastes, and reduced the BOD from 30,000 mg/l to 15 mg/l. An oxidation ditch was used the U.S.A. (Smith & Hazen, 1967) for secondary treatment of piggery wastes after a period in an aerobic lagoon. The BOD of the waste was reduced from 4,800 mg/l to 800 mg/l in the lagoon and then to 60 mg/l in the oxidation ditch. However, in the United Kingdom, purification of piggery wastes by treatment in an oxidation ditch (Pontin & Baxter, 1968) resulted only in the BOD being reduced from 2,320 mg/l to 590 mg/l. A general

disadvantage of the activated sludge process is that its efficiency is reduced by sudden changes in the nature of the waste, and its recovery may be prolonged. Therefore, since piggery wastes are highly variable in their nature (Table 1) it would suggest that the activated sludge process may be impractical for their purification. Difficulties which have particularly applied to oxidation ditches have been excessive foaming, freezing of the rotors in winter, and the formation of banks of undigested fibre (Pontin & Baxter, 1968). A water spray was found to be adequate for controlling foaming in the ditch investigated by Scheltinga (1966), and since it diluted the waste it might explain the high degree of purification which was achieved. However, despite its disadvantages, it is generally agreed (Scheltinga, 1966; Smith & Hazen, 1968; Pontin & Baxter, 1968) that the Pasveer oxidation ditch may possibly be used successfully for the purification of piggery wastes.

The third method of aerobic waste treatment is by trickling filters, the waste irrigating a support medium whose surfaces are colonized by a film of microorganisms. The great expense involved in installing conventional stone-packed filter beds restrict their serious consideration as a means of treating wastes on the farm. However, the innovation of geometrically ordered plastic filters has presented a more convenient application of trickling filters. Because of their low bulk density they can be used in tall towers which have no requirement for massive support structures near for excavation, and they occupy a smaller area that conventional filter beds of equivalent capacity. The principal plastic media are Flocor (I.C.I. (Hyde) Ltd., P.O. Box 19, Templar House, 81-87 High Holborn, London W.C.1.), Surfpac (Hydronyl Ltd., 14 Gloucester Road, London S.W.7.) and Cloisonyle (Cegedur, 89 Rue de

Tocqueville, Paris XVIII^{e}, France). Although these plastic packings have been investigated for treatment of domestic sewage (Askew, 1966, 1967; Eden, Truesdale & Mann, 1966) they were designed primarily for partial purification of wastes at high hydraulic and organic loadings. Wastes which have been treated successfully include those originating from paper production and phenol refining (Bryan & Moeller, 1960), and from whisky distilling, fruit and vegetable canning, brewing, textile dyeing and finishing, yeast production and synthetic resin production (Askew, 1966, 1967). These results seem to suggest that plastic media may be useful for the purification of piggery wastes but no published report of their application to this problem has been located. However, a 12 ft high tower, half fitted with 1 ft square wooden battens $1\frac{1}{2}$ in apart and the remaining space filled with porous aggregate, has been used for the purification of piggery wastes at the National Institute of Agricultural Engineering, Silsoe, Beds. Pig slurry was circulated continuously at 700gal/h through the tower, and it has been claimed in a semi-popular report (Hope, 1970) that the BOD of the waste was reduced from 80,000 mg/l to 200 mg/l. Because of this absence of information on the suitability of trickling filters for purification of piggery wastes, it was hoped that the present investigation of experimental trickling filters (Gloyna et al., 1952) might give some indication of their value. Retention of a soluble tracer and the degree of purification of domestic sewage in this apparatus was very much the same as that in a 7 ft plastic-packed tower irrigated at equivalent hydraulic loadings per wetted surface area (Eden et al., 1966). However, it was not possible to initiate film development by irrigation with piggery wastes, even when diluted to 2,000 mg/l COD. Highly diluted piggery wastes (1,500 mg/l COD)

could be purified on films developed by irrigation with domestic sewage but stronger wastes caused changes in the microflora and the film to slough. This would suggest that unless the wastes are pre treated trickling filters may be unsuitable for the purification of piggery wastes.

Anaerobic biological methods of disposal

Anaerobic breakdown of organic matter proceeds in two stages, 1) volatile fatty acids are produced by fermentation of the organic substrates, and 2) the fatty acids are broken down to yield methane. Since the methane-forming bacteria grow best between pH6.4 and 7.8 and production of volatile fatty acids in the first phase of anaerobic breakdown can result in pH 4.0 to 6.5 (Jenkins, 1963) successful anaerobic digestion depends on balancing the two phases. Anaerobic digestion is frequently used to supplement other methods of waste purification. Septic tanks, and their derivatives, are a common means of depositing and/or liquifying suspended solids in order to make wastes more suitable for aerobic treatment. Anaerobic decomposition in a septic tank causes bubbles of gas to rise to the surface, solid particles which are carried by the bubbles form a scum on the surface and help to keep the air from direct contact with the waste.

A modification of the septic tank (the hydrolytic tank) is divided into compartments by vertical spines (colloiders) to which colloids and fine suspended particles adhere, and subsequently, flake off to the bottom of the chamber. Anaerobic digestion is also used to dispose of the sludge which results from screening, settling and aerobic purification of wastes. It is carried out in enclosed tanks provided with gas collection facilities and with provision for the withdrawal of digested sludge, at normal ambient temperatures sewage sludge requires 3 to 6 months for complete digestion

(Jenkins, 1963). However, it is usual to use the methane for heating the sludge to about 35° so that digestion can be completed in around 30 days(Baines, 1970).

Anaerobic digestion of piggery wastes have been investigated by several workers (Jeffrey, & Ricketts, 1965; Baines, 1970; Hobson & Shaw, 1971) and, in terms of gas production and analysis, has compared well with domestic sludge digestion (Jeffrey et al; 1965; Hobson & Shaw, 1971). Therefore it appears likely that a satisfactory methane fermentation of piggery wastes can be established and maintained but the literature available on its practical application is scarce. The cost of installation of anaerobic digesters may be high and in operation there may be difficulty in balancing the two phases of the process.

Conclusions

The information which is available on the biological treatment of piggery wastes is still too scarce to permit recommendations. However, it is evident that aerobic processes have been designed for dilute wastes whereas an aerobic digestion has been used mainly for treating sludges. Although there is no general agreement as to the nature and composition of piggery wastes they have a greater resemblance to sludge than to sewage. So it is tempting to suggest that anaerobic digestion of piggery wastes might yield an effluent which would be amenable to subsequent aerobic biological treatment. However it has been indicated in the present study that piggery wastes may not be amenable to aerobic purification unless the concentration of organic matter is below a certain level. It is unlikely that the effluent from anaerobic digestion (Hobson & Shaw, 1971) could be purified aerobically without further dilution. The amount of water required for this purpose would seem

to rule out trickling filter for aerobic purification subsequent to anaerobic digestion, but it is possible that by controlling the flow-through rate in the activated sludge process the concentration of organic matter in the reactor (tank or ditch etc.) might be maintained below a critical level.

Therefore it is tempting to suggest that purification of piggery wastes can be achieved by anaerobic digestion followed by aerobic treatment of the effluent using the activated sludge process (most likely the Pasveer oxidation ditch). Such a set-up is likely to be expensive in terms of initial cost and labour required to supervise the waste treatment processes. Although the problem of disposal of piggery wastes is likely to be confined to large intensive enterprises it may be that the only economical solution will lie in cooperative waste treatment plants serving several farms in the same locality.

APPENDICES

APPENDIX 1

Irrigation of experimental trickling filters with settled domestic sewage obtained from Bath Corporation Sewage Treatment Works

The results from every film during the first four days of irrigation were used to prepare Fig. 5

The results from film No. 4 were used to prepare Fig. 6

The films used in bacteriological analyses were Nos. 5, 6 and 7.

Films Nos. 9 & 10 irrigated with wastes with CODs between 400 and 700 mg/l were used to prepare Fig. 12

Films Nos. 11 & 12 were used to prepare Fig. 11

No. 1

Day	0	1	2	3	4	5	6
Weight of Film (g)	0	2·1	6·3	14·0	32·0	. .	. .
Waste COD (mg/l)	392	384	448	456	392	. .	. .
% COD reduction	1·3	7·5	21·5	42·0	. .	. .	. .

Day	7	8	9	10	11
Weight of Film	28·9	30·4	36·2	34·7	35·8
Waste COD (mg/l)	386	420	510	520	640
% COD reduction	54·0	58·0	60·0	62·0	. .

No. 2

Day	0	1	2	3	4	5	6
Weight of Film (g)	0	1·9	3·2	17·2	31·8	. .	. .
Waste COD (mg/l)	436	296	396	520	. .	. .	. .
% COD reduction	2·0	7·0	23·0	58·0	. .	. .	. .

Day	7	8	9	10	11
Weight of Film (g)	42·2	45·3	37·2	35·2	40·4
Waste COD (mg/l)	386	640	520	420	510
% COD reduction	56·0	60·5	62·0	57·0	. .

No. 3

Day	0	1	2	3	4
Weight of Film (g)	0	1·8	5·2	14·6	41·2
Waste COD (mg/l)	320	610	484	520	. .
% COD reduction	0·5	7·5	24·0	48·3	. .

No. 4

Day	0	1	2	3	4	5	6
Weight of Film (g)	0	3·8	10·0	20·0	28·4	28·4	42·0
Waste COD (mg/l)	368	420	360	480	432	510	. .
% COD reduction	0·5	15·0	30·2	69·4	60·4	. .	. .

Day	7	8	9	10	11	12	13
Weight of Film (g)	49·1	54·5	57·2	63·4	68·5	. .	. .
Waste COD (mg/l)	560	384	512	298	468	. .	. .
% COD reduction	61·0	53·0	60·0	50·0	. .	. .	. .

Day	14	15	16	17	18	19	20
Weight of Film (g)	73·5	76·5	72·4	79·5	80·8	. .	. .
Waste COD (mg/l)	400	360	832	440	452	. .	. .
% COD reduction	57·5	53·0	75·0	56·0	. .	. .	. .

Day	21	22	23	24	25	26	27
Weight of Film (g)	88·1	90·6	84·3	87·5	63·1	. .	. .
Waste COD (mg/l)	332	540	440	604	620	. .	. .
% COD reduction	51·5	60·0	57·5	62·0	. .	. .	. .

Day	28	29	30	31
Weight of Film (g)	61·2	72·1	70·4	63·2
Waste COD (mg /l)	520	680	540	385
% COD reduction	60·0	65·0	61·2	53·5

No. 5

Day	0	1	2	3
Weight of Film (g)	0	3·4	6·8	18·4
Waste COD (mg/l)	620	710	580	610
% COD reduction	1·4	10·2	27·0	38·5

No. 6

Day	0	1	2	3
Weight of Film (g)	0	1·1	5·0	16·5
Waste COD (mg/l)	280	460	485	630
% COD reduction	0	5·0	18·0	49·2

No. 7

Day	0	1	2	3	4	5	6
Weight of Film (g)	0	4·8	9·2	20·8	43·7	. .	. .
Waste COD (mg/l)	280	560	484	630	616	. .	. .
% COD reduction	0·5	22·0	24·0	64·0	. .	. .	. .

Day	7	8	9	10	11	12	13
Weight of Film (g)	52·3	58·4	60·0	57·2	53·1	. .	. .
Waste COD (mg/l)	380	484	630	560	580	. .	. .
% COD reduction	48·0	60·0	66·0	62·6	. .	. .	. .

Day	14	15	16	17	18	19	20
Weight of Film (g)	56·2	49·8	47·5	52·4	57·2	59·1	56·2
Waste COD (mg/l)	448	456	392	480	520	. .	. .
% COD reduction	55·0	56·5	49·0	60·0	. .	. .	. .

No. 8

Day	0	1	2	3	4	5	6
Weight of Film (g)	0	4·2	9·0	19·1	37·9	. .	. .
Waste COD (mg/l)	520	380	512	510	. .	. .	. .
% COD reduction	1·8	17·0	22·0	54·2	. .	. .	. .

Day	7	8	9
Weight of Film (g)	42·2	39·6	38·7
Waste COD (mg/l)	620	380	. .
% COD reduction	64·0	49·0	. .

No. 9

Day	0	1	2	3	4	5	6
Weight of Film (g)	0	2·7	5·2	14·8	26·8	. .	. .
Waste COD (mg/l)	520	560	480	610	. .	. .	. .
% COD reduction	1·5	10·1	26·6	46·4	. .	. .	. .

Day	7	8	9	10	11	12	13
Weight of Film (g)	34·8	40·2	48·5	45·8	49·9	. .	. .
Waste COD (mg/l)	480	380	364	520	480	. .	. .
% COD reduction	60·8	51·0	54·3	62·0	. .	. .	. .

Day	14	15	16	17	18	19
Weight of Film (g)	71·5	79·2	70·8	63·0	60·1	54·3
Waste COD (mg/l)	374	610	384	480	510	610
% COD reduction	52·0	64·0	45·0	53·2	61·7	56·9

No. 11

Day	0	1	2	3	4	5	6
Weight of Film (g)	0	3·2	7·0	18·8	32·0	28·6	38·9
Waste COD (mg/l)	468	476	510	520	468	. .	. .
% COD reduction	1·2	9·8	20·0	61·2	. .	. .	. .

Day	7	8	9	10	11	12	13
Weight of Film (g)	38·1	51·1	60·1	63·3	76·6	72·1	. .
Waste COD (mg/l)	240	400	836	560	640	. .	. .
% COD reduction	46·5	57·0	79·2	57·0	59·1	. .	. .

Day	14	15	16	17
Weight of Film (g)	67·2	63·4	68·1	73·4
Waste COD (mg/l)	280	374	744	640
% COD reduction	41·5	52·5	74·3	69·2

No. 12

Day	0	1	2	3	4	5	6
Weight of Film (g)	0	3·6	8·4	18·6	37·5	. .	. .
Waste COD (mg/l)	468	476	510	420	468	. .	. .
% COD reduction	1·6	12·0	29·0	57·3	. .	. .	. .

Day	7	8	9	10	11	12	13
Weight of Film (g)	28·3	29·2	22·6	31·4	29·0	32·3	. .
Waste COD (mg/l)	240	400	836	560	640	. .	. .
% COD reduction	54·0	53·0	76·4	64·6	65·1	. .	. .

Day	14	15	16	17	18	19	20
Weight of Film (g)	34·2	37·8	31·6	29·1	32·9	38·4	41·2
Waste COD (mg/l)	280	374	744	640	. .	. .	. .
% COD reduction	48·5	49·9	71·1	67·8	. .	. .	. .

No. 13

Day	0	1	2	3	4	5
Weight of Film (g)	0	5·1	9·1	18·6	36·6	48·2
Waste COD (mg/l)	320	320	410	384	. .	. .
% COD reduction	1·2	17·0	29·0	54·1	. .	. .

APPENDIX 2

Irrigation of experimental trickling filters with piggery waste or diluted piggery waste

Film No. 8/11 was used to prepare Fig. 11 and Film No. 9/12 was used for the bacteriological analysis presented in Fig. 10.

No. 1. Piggery Waste on clean tube

Day	0	1	2	3	4	5	6
Weight of Film (g)	0	0·1	6·1	8·7	9·1	. .	. .
Waste COD (mg/l)	———		8-9,000		———		
% COD reduction	———		NIL		———		

Day	7	8	9
Weight of Film (g)	8·2	9·0	8·7
Waste COD (mg/l)	——	8-9,000	———
% COD reduction	——	NIL	———

No. 2. Piggery Waste on a clean tube

Day	0	1	2	3	4	5	6
Weight of Film (g)	0	0·5	8·7	9·1	9·3	. .	. .
Waste COD (mg/l)	———		8-9,000		———		
% COD reduction	———		NIL		———		

Day	7	8	9
Weight of Film (g)	9·1	9·0	8·3
Waste COD (mg/l)	——	8-9,000	———
% COD reduction	——	NIL	———

No. 3. Diluted piggery waste on a clean tube

Day	0	1	2	3	4	5	6
Weight of Film (g)	0	0·5	7·1	10·8	11·9	. .	. .
Waste COD (mg/l)	———		2,000		———		
% COD reduction	———		NIL		———		

Day	7	8	9
Weight of Film (g)	11·5	10·9	11·1
Waste COD (mg/l)	——	2,000	———
% COD reduction	——	NIL	———

No. 4 Diluted piggery waste on a clean tube

Day	0	1	2	3	4	5	6
Weight of Film (g)	0	0·7	5·3	10·5	11·5	. .	. .
Waste COD (mg/l)	———		2,000		———		
% COD reduction	———		NIL		———		

Day	7	8	9
Weight of Film (g)	11·1	10·4	10·7
Waste COD (mg/l)	——	2,000	———
% COD reduction	——	NIL	———

No. 5/8 Diluted piggery waste applied to a film (No.8) developed in domestic sewage

Day	0	1	2	3	4	5	6
Weight of Film (g)	38·7	32·3	11·6	. .	. .	9·8	10·6
Waste COD (mg/l)	———		2,000		———		
% COD reduction	———		NIL		———		

No. 6/9 Diluted piggery waste applied to a film (No. 9) developed in domestic sewage

Day	0	1	2	3	4	5	6
Weight of Film (g)	58·5	15·8	12·2	13·9	11·7	. .	. .
Waste COD (mg/l)	———		4,000		———		
% COD reduction	———		NIL		———		

No. 7/10 Piggery waste applied to a film (No.10) developed in domestic sewage

Day	0	1	2	3	4	5	6
Weight of Film (g)	49·3	21·2	7·6	6·2	9·4	. .	. .
Waste COD (mg/l)	———		8-9,000		———		
% COD reduction	———		NIL		———		

No. 8/11 Increasing concentrations of dilute piggery waste applied to a film (No.11) developed on domestic sewage

Day	0	1	2	3	4	5	6
Weight of Film (g)	68·7	60·6	59·1	62·6	39·8	46·0	52·7
Waste COD (mg/l)	280	332	324	360	420	388	604
% reduction	50·0	60·0	56·8	52·0	50·0	47·5	48·4
Day	7	8	9	10	11	12	13
Weight of Film (g)	58·3	. .	. .	58·1	60·4	65·6	67·8
Waste COD (mg/l)	. .	. .	. .	788	768	770	788
% reduction	. .	. .	. .	51·0	49·0	50·5	52·0
Day	14	15	16	17	18	19	20
Weight of Film (g)	75·0	. .	. .	68·0	62·9	52·2	46·8
Waste COD (mg/l)	. .	. .	. .	464	828	1032	1032
% reduction	. .	. .	. .	59·5	42·0	51·0	49·0
Day	21	22	23	24	25	26	27
Weight of Film (g)	56·5	. .	. .	48·9	54·9	47·4	51·7
Waste COD (mg/l)	. .	. .	. .	1232	1028	1148	1500
% reduction	. .	. .	. .	42·0	49·0	47·5	45·0
Day	28	29	30	31	32	33	34
Weight of Film (g)	49·2	. .	. .	39·7	42·8	47·1	18·3
Waste COD (mg/l)	. .	. .	. .	2432	2016	2600	3224
% reduction	. .	. .	. .	8·5	14·0	10·0	NIL
Day	35	36	37	38	39	40	41
Weight of Film (g)	10·1	9·8	8·3	9·1			
Waste COD (mg/l)	4000	4000	. .	. .			
% reduction	NIL	NIL	. .	. .			

No. 9/12 Increasing concentrations of diluted piggery waste applied to a film (No.12) developed on domestic sewage

Day	0	1	2	3	4	5	6
Weight of Film (g)	46·1	47·4	53·7	62·4	61·2	. .	. .
Waste COD (mg/l)	340	420	388	604	560	. .	. .
% reduction	62·4	53·4	33·0	56·0	. .	. .	. .

Day	7	8	9	10	11	12	13
Weight of Film (g)	56·7	53·1	50·1	65·5	61·5	64·7	. .
Waste COD (mg/l)	640	560	680	768	788	. .	. .
% reduction	52·0	49·0	47·5	51·5	. .	. .	...

APPENDIX 3

Competition between Acinetobacter 4 and Pseudomonas putida in Synthetic Sewage medium

Growth of Acinetobacter 4 in standing flasks of Synthetic Sewage Medium

Experiment 1			Experiment 2		
Time (h)	Viable Count ml on TA	Mean	Time (h)	Viable Count ml on TA	Mean
0	$3{\cdot}2 \times 10^5$ $2{\cdot}0 \times 10^5$ $1{\cdot}8 \times 10^5$	$2{\cdot}3 \times 10^5$	0	$4{\cdot}0 \times 10^5$ $2{\cdot}8 \times 10^5$ $2{\cdot}0 \times 10^5$	$2{\cdot}9 \times 10^5$
2·0	$2{\cdot}6 \times 10^5$ $2{\cdot}0 \times 10^5$ $1{\cdot}8 \times 10^5$	$2{\cdot}1 \times 10^5$	2	$4{\cdot}0 \times 10^5$ $3{\cdot}2 \times 10^5$ $2{\cdot}2 \times 10^5$	$3{\cdot}1 \times 10^5$
3·25	$2{\cdot}6 \times 10^5$ $2{\cdot}4 \times 10^5$ $2{\cdot}0 \times 10^5$	$2{\cdot}65 \times 10^5$	8	$3{\cdot}2 \times 10^6$ $2{\cdot}8 \times 10^6$ $2{\cdot}2 \times 10^6$	$2{\cdot}7 \times 10^6$
4·5	$4{\cdot}0 \times 10^5$ $3{\cdot}8 \times 10^5$ $3{\cdot}0 \times 10^5$	$3{\cdot}6 \times 10^5$	12	$1{\cdot}4 \times 10^7$ $1{\cdot}0 \times 10^7$ $9{\cdot}0 \times 10^6$	$1{\cdot}1 \times 10^7$
5·25	$5{\cdot}6 \times 10^5$ $3{\cdot}8 \times 10^5$ $3{\cdot}0 \times 10^5$	$4{\cdot}1 \times 10^5$			
6·75	$1{\cdot}2 \times 10^6$ $7{\cdot}0 \times 10^5$ $6{\cdot}6 \times 10^5$	$8{\cdot}2 \times 10^5$			
7·5	$1{\cdot}8 \times 10^6$ $1{\cdot}0 \times 10^6$ $0{\cdot}8 \times 10^6$	$1{\cdot}2 \times 10^6$			
9·0	$4{\cdot}2 \times 10^6$ $2{\cdot}8 \times 10^6$ $2{\cdot}0 \times 10^6$	$3{\cdot}0 \times 10^6$			
16·0	$6{\cdot}4 \times 10^7$ $5{\cdot}0 \times 10^7$ $4{\cdot}4 \times 10^7$	$5{\cdot}6 \times 10^7$			

Growth of Pseudomonas putida in standing flasks of Synthetic Sewage Medium

Experiment 1			Experiment 2		
Time (h)	Viable Count/ ml on GMS	Mean	Time (h)	Viable Count/ ml on GMS	Mean
0	$3{\cdot}4 \times 10^5$ $2{\cdot}6 \times 10^5$ $1{\cdot}8 \times 10^5$	$2{\cdot}65 \times 10^5$	0	$3{\cdot}2 \times 10^5$ $2{\cdot}6 \times 10^5$ $2{\cdot}4 \times 10^5$	$2{\cdot}7 \times 10^5$
1·5	$4{\cdot}4 \times 10^5$ $2{\cdot}8 \times 10^5$ $2{\cdot}4 \times 10^5$	$3{\cdot}2 \times 10^5$	3·0	$8{\cdot}2 \times 10^5$ $8{\cdot}0 \times 10^5$ $7{\cdot}8 \times 10^5$	$8{\cdot}0 \times 10^5$
4·5	$3{\cdot}0 \times 10^6$ $2{\cdot}0 \times 10^6$ $8{\cdot}0 \times 10^5$	$1{\cdot}9 \times 10^6$	6·5	$5{\cdot}8 \times 10^7$ $4{\cdot}6 \times 10^7$ $4{\cdot}0 \times 10^7$	$4{\cdot}8 \times 10^7$
5·75	$6{\cdot}8 \times 10^6$ $5{\cdot}6 \times 10^6$ $4{\cdot}4 \times 10^6$	$5{\cdot}6 \times 10^6$	9·0	$3{\cdot}6 \times 10^9$ $3{\cdot}2 \times 10^9$ $2{\cdot}8 \times 10^9$	$3{\cdot}1 \times 10^9$
6·75	$4{\cdot}0 \times 10^7$ $3{\cdot}4 \times 10^7$ $3{\cdot}4 \times 10^7$	$3{\cdot}55 \times 10^7$	12·0	$4{\cdot}2 \times 10^9$ $3{\cdot}6 \times 10^9$ $3{\cdot}4 \times 10^9$	$3{\cdot}7 \times 10^9$
8·5	$1{\cdot}4 \times 10^9$ $1{\cdot}4 \times 10^9$ $9{\cdot}0 \times 10^8$	$1{\cdot}2 \times 10^9$			
10·0	$7{\cdot}0 \times 10^9$ $7{\cdot}0 \times 10^9$ $5{\cdot}2 \times 10^9$	$6{\cdot}4 \times 10^9$			
16·0	$6{\cdot}8 \times 10^8$ $6{\cdot}4 \times 10^8$ $6{\cdot}2 \times 10^8$	$6{\cdot}4 \times 10^8$			

Growth of Acinetobacter 4 and Pseudomonas putida together in standing cultures of Synthetic Sewage medium

Experiment 1

Time (h)	Viable Count/ ml on TA	Mean	Viable Count/ ml on GMS	Mean
0	6.8×10^5 5.6×10^5 3.6×10^5	5.3×10^5	4.2×10^5 4.2×10^5 3.4×10^5	3.9×10^5
2.0	6.0×10^5 4.2×10^5 4.0×10^5	4.7×10^5	5.6×10^5 3.8×10^5 3.2×10^5	4.2×10^5
4.0	3.1×10^6 1.8×10^6 9.4×10^5	1.95×10^6	2.8×10^6 2.0×10^6 1.6×10^6	2.1×10^6
6.0	9.0×10^6 7.8×10^6 7.8×10^6	8.2×10^6	6.8×10^6 5.4×10^6 5.2×10^6	5.8×10^6
7.5	4.8×10^7 4.6×10^7 4.4×10^7	4.6×10^7	5.0×10^7 5.0×10^7 3.2×10^7	4.4×10^7
9.0	3.2×10^9 2.8×10^9 1.2×10^9	2.4×10^9	3.2×10^9 2.6×10^9 2.6×10^9	2.8×10^9
11.0	5.2×10^9 5.0×10^9 3.6×10^9	4.6×10^9	7.2×10^9 5.2×10^9 5.0×10^9	5.8×10^9
16.0	1.0×10^9 9.8×10^8 7.8×10^8	9.7×10^8	9.4×10^8 9.4×10^8 8.8×10^8	9.2×10^8

Growth of Acinetobacter 4 and Pseudomonas putida together in standing flasks of Synthetic Sewage medium

Experiment 2

Time (h)	Viable Count/ ml on TA	Mean	Viable Count/ ml on GMS	Mean
0	$6{\cdot}2 \times 10^5$ $5{\cdot}8 \times 10^5$ $5{\cdot}4 \times 10^5$	$5{\cdot}8 \times 10^5$	$3{\cdot}2 \times 10^5$ $3{\cdot}2 \times 10^5$ $2{\cdot}0 \times 10^5$	$2{\cdot}8 \times 10^5$
4·0	$2{\cdot}2 \times 10^6$ $1{\cdot}6 \times 10^6$ $1{\cdot}6 \times 10^6$	$1{\cdot}8 \times 10^6$	$2{\cdot}2 \times 10^6$ $1{\cdot}6 \times 10^6$ $1{\cdot}4 \times 10^6$	$1{\cdot}7 \times 10^6$
8·0	$5{\cdot}8 \times 10^7$ $4{\cdot}4 \times 10^7$ $4{\cdot}2 \times 10^7$	$4{\cdot}8 \times 10^7$	$5{\cdot}6 \times 10^6$ $5{\cdot}2 \times 10^7$ $4{\cdot}0 \times 10^7$	$4{\cdot}9 \times 10^7$
12·0	$5{\cdot}8 \times 10^9$ $5{\cdot}0 \times 10^9$ $4{\cdot}8 \times 10^9$	$5{\cdot}2 \times 10^9$	$6{\cdot}0 \times 10^9$ $5{\cdot}4 \times 10^9$ $5{\cdot}4 \times 10^9$	$5{\cdot}6 \times 10^9$

Growth of Acinetobacter 4 in aerated flasks of Synthetic Sewage medium

Experiment 1			Experiment 2		
Time (h)	Viable Count/ ml on TA	Mean	Time (h)	Viable Count/ ml on TA	Mean
0	$2{\cdot}8 \times 10^5$ $2{\cdot}0 \times 10^5$ $2{\cdot}0 \times 10^5$	$2{\cdot}3 \times 10^5$	0	$3{\cdot}8 \times 10^5$ $2{\cdot}2 \times 10^5$ $2{\cdot}2 \times 10^5$	$2{\cdot}7 \times 10^5$
1·5	$7{\cdot}0 \times 10^5$ $3{\cdot}8 \times 10^5$ $3{\cdot}4 \times 10^5$	$4{\cdot}74 \times 10^5$	3·0	$1{\cdot}1 \times 10^7$ $9{\cdot}8 \times 10^6$ $7{\cdot}2 \times 10^6$	$9{\cdot}1 \times 10^6$
3·0	$9{\cdot}4 \times 10^6$ $9{\cdot}4 \times 10^6$ $7{\cdot}2 \times 10^6$	$8{\cdot}7 \times 10^6$	9·0	$7{\cdot}0 \times 10^8$ $5{\cdot}6 \times 10^8$ $3{\cdot}2 \times 10^8$	$5{\cdot}6 \times 10^8$
4·5	$2{\cdot}8 \times 10^7$ $2{\cdot}2 \times 10^7$ $2{\cdot}0 \times 10^7$	$2{\cdot}3 \times 10^7$	12·0	$4{\cdot}2 \times 10^9$ $4{\cdot}2 \times 10^9$ $4{\cdot}2 \times 10^9$	$4{\cdot}2 \times 10^9$
6·0	$1{\cdot}6 \times 10^8$ $1{\cdot}4 \times 10^8$ $7{\cdot}2 \times 10^7$	$9{\cdot}4 \times 10^7$			
8·0	$7{\cdot}2 \times 10^8$ $4{\cdot}8 \times 10^8$ $4{\cdot}0 \times 10^8$	$5{\cdot}3 \times 10^8$			
10·5	$4{\cdot}6 \times 10^9$ $3{\cdot}6 \times 10^9$ $3{\cdot}2 \times 10^9$	$3{\cdot}8 \times 10^9$			
16·0	$2{\cdot}8 \times 10^9$ $2{\cdot}8 \times 10^9$ $2{\cdot}6 \times 10^9$	$2{\cdot}78 \times 10^9$			

Growth of Pseudomonas putida in aerated flasks of Synthetic Sewage medium

Experiment 1			Experiment 2		
Time (h)	Viable Count/ ml on GMS	Mean	Time (h)	Viable Count/ ml on GMS	Mean
0	1.8×10^5 1.2×10^5 6.0×10^4	1.2×10^5	0	2.8×10^5 1.8×10^5 1.8×10^5	2.1×10^5
2.0	1.6×10^5 1.4×10^5 1.2×10^5	1.4×10^5	3.0	2.2×10^5 1.8×10^5 1.4×10^5	1.8×10^5
4.0	1.4×10^6 9.8×10^5 5.4×10^5	9.7×10^5	9.0	1.3×10^9 8.4×10^8 6.2×10^8	9.2×10^8
5.0	8.6×10^6 8.0×10^6 8.0×10^6	8.2×10^6	12.0	7.2×10^9 7.2×10^9 6.4×10^9	6.9×10^9
7.0	1.1×10^9 8.4×10^8 8.2×10^8	9.2×10^8			
10.0	8.8×10^9 8.8×10^9 8.8×10^9	8.8×10^9			
12.0	6.8×10^9 6.2×10^9 6.2×10^9	6.4×10^9			
16.0	9.8×10^8 9.6×10^8 8.2×10^8	9.2×10^8			

Growth of Acinetobacter 4 and Pseudomonas putida together in aerated flasks of Synthetic Sewage medium

Experiment 1

Time (h)	Viable Count/ ml on TA	Mean	Viable Count/ ml on GMS	Mean
0	$5{\cdot}4 \times 10^5$ $5{\cdot}0 \times 10^5$ $5{\cdot}0 \times 10^5$	$5{\cdot}1 \times 10^5$	$4{\cdot}2 \times 10^5$ $4{\cdot}0 \times 10^5$ $3{\cdot}6 \times 10^5$	$3{\cdot}9 \times 10^5$
2·5	$5{\cdot}2 \times 10^5$ $4{\cdot}0 \times 10^5$ $3{\cdot}8 \times 10^5$	$4{\cdot}3 \times 10^5$	$1{\cdot}3 \times 10^5$ $1{\cdot}2 \times 10^5$ $1{\cdot}1 \times 10^5$	$1{\cdot}23 \times 10^5$
4·0	$4{\cdot}8 \times 10^6$ $4{\cdot}2 \times 10^6$ $3{\cdot}4 \times 10^6$	$4{\cdot}1 \times 10^6$	$1{\cdot}2 \times 10^5$ $1{\cdot}1 \times 10^5$ $1{\cdot}1 \times 10^5$	$1{\cdot}15 \times 10^5$
6·0	$2{\cdot}4 \times 10^7$ $1{\cdot}2 \times 10^7$ $1{\cdot}2 \times 10^7$	$1{\cdot}6 \times 10^7$	$1{\cdot}6 \times 10^5$ $1{\cdot}4 \times 10^5$ $9{\cdot}8 \times 10^4$	$1{\cdot}25 \times 10^5$
7·5	$2{\cdot}2 \times 10^8$ $2{\cdot}2 \times 10^8$ $2{\cdot}0 \times 10^8$	$2{\cdot}1 \times 10^8$	$2{\cdot}4 \times 10^5$ $1{\cdot}4 \times 10^5$ $1{\cdot}4 \times 10^5$	$1{\cdot}7 \times 10^5$
8·5	$1{\cdot}1 \times 10^9$ $9{\cdot}8 \times 10^8$ $7{\cdot}4 \times 10^8$	$9{\cdot}4 \times 10^8$	$2{\cdot}8 \times 10^5$ $2{\cdot}2 \times 10^5$ $2{\cdot}0 \times 10^5$	$2{\cdot}3 \times 10^5$
10·0	$5{\cdot}4 \times 10^9$ $4{\cdot}4 \times 10^9$ $4{\cdot}4 \times 10^9$	$4{\cdot}7 \times 10^9$	$2{\cdot}8 \times 10^5$ $1{\cdot}6 \times 10^5$ $1{\cdot}4 \times 10^5$	$1{\cdot}9 \times 10^5$
16·0	$5{\cdot}4 \times 10^9$ $5{\cdot}6 \times 10^9$ $5{\cdot}2 \times 10^9$	$5{\cdot}5 \times 10^9$	$6{\cdot}2 \times 10^5$ $5{\cdot}2 \times 10^5$ $4{\cdot}2 \times 10^5$	$5{\cdot}2 \times 10^5$

Growth of Acinetobacter 4 and Pseudomonas putida together in aerated flasks of Synthetic Sewage medium

Experiment 2

Time (h)	Viable Count/ ml on TA	Mean	Viable Count/ ml on GMS	Mean
0	$6{\cdot}2 \times 10^5$ $5{\cdot}4 \times 10^5$ $5{\cdot}0 \times 10^5$	$5{\cdot}5 \times 10^5$	$2{\cdot}4 \times 10^5$ $2{\cdot}4 \times 10^5$ $2{\cdot}0 \times 10^5$	$2{\cdot}4 \times 10^5$
4·0	$4{\cdot}0 \times 10^6$ $3{\cdot}8 \times 10^6$ $3{\cdot}6 \times 10^6$	$3{\cdot}8 \times 10^6$	$1{\cdot}1 \times 10^5$ $7{\cdot}4 \times 10^4$ $7{\cdot}2 \times 10^4$	$8{\cdot}5 \times 10^4$
8·0	$5{\cdot}2 \times 10^8$ $5{\cdot}2 \times 10^8$ $4{\cdot}6 \times 10^8$	$5{\cdot}0 \times 10^8$	$2{\cdot}6 \times 10^5$ $2{\cdot}2 \times 10^5$ $1{\cdot}6 \times 10^5$	$2{\cdot}1 \times 10^5$
12·0	$4{\cdot}6 \times 10^9$ $4{\cdot}6 \times 10^9$ $4{\cdot}6 \times 10^9$	$4{\cdot}6 \times 10^9$	$3{\cdot}4 \times 10^5$ $3{\cdot}2 \times 10^5$ $2{\cdot}8 \times 10^5$	$3{\cdot}1 \times 10^5$

APPENDIX 4

Growth of the Yellow-pigmented Gram-negative rod with Acinetobacter 4 or Pseudomonas putida in Synthetic Sewage medium

Growth of a Yellow-pigmented Gram-negative rod with Acinetobacter 4 in aerated flasks of Synthetic Sewage medium

Experiment 1

Time (h)	Non pigmented Viable Cont/ml	Mean	Pigmented Viable Cont/ml	Mean
0	$2{\cdot}4 \times 10^5$ $2{\cdot}0 \times 10^5$ $2{\cdot}0 \times 10^5$	$2{\cdot}0 \times 10^5$	$2{\cdot}3 \times 10^4$ $1{\cdot}9 \times 10^4$ $9{\cdot}2 \times 10^3$	$1{\cdot}7 \times 10^4$
4·0	$7{\cdot}2 \times 10^5$ $7{\cdot}0 \times 10^5$ $5{\cdot}4 \times 10^5$	$6{\cdot}5 \times 10^5$	$5{\cdot}2 \times 10^4$ $3{\cdot}8 \times 10^4$ $1{\cdot}0 \times 10^4$	$3{\cdot}3 \times 10^4$
6·0	$5{\cdot}2 \times 10^6$ $5{\cdot}0 \times 10^6$ $4{\cdot}8 \times 10^6$	$5{\cdot}0 \times 10^6$	$4{\cdot}8 \times 10^4$ $4{\cdot}2 \times 10^4$ $4{\cdot}0 \times 10^4$	$4{\cdot}3 \times 10^4$
8·0	$8{\cdot}4 \times 10^6$ $8{\cdot}2 \times 10^6$ $7{\cdot}0 \times 10^6$	$8{\cdot}0 \times 10^6$	$3{\cdot}0 \times 10^5$ $3{\cdot}0 \times 10^5$ $3{\cdot}0 \times 10^5$	$3{\cdot}0 \times 10^5$
10·0	$5{\cdot}2 \times 10^7$ $3{\cdot}6 \times 10^7$ $3{\cdot}6 \times 10^7$	$4{\cdot}1 \times 10^7$	$9{\cdot}0 \times 10^6$ $8{\cdot}6 \times 10^6$ $8{\cdot}0 \times 10^6$	$8{\cdot}5 \times 10^6$
12·0	$5{\cdot}2 \times 10^8$ $5{\cdot}0 \times 10^8$ $4{\cdot}8 \times 10^8$	$5{\cdot}0 \times 10^8$	$1{\cdot}8 \times 10^8$ $1{\cdot}5 \times 10^8$ $1{\cdot}1 \times 10^8$	$1{\cdot}45 \times 10^8$
16·0	$4{\cdot}6 \times 10^8$ $4{\cdot}0 \times 10^8$ $4{\cdot}0 \times 10^8$	$4{\cdot}2 \times 10^8$	$2{\cdot}6 \times 10^8$ $1{\cdot}8 \times 10^8$ $1{\cdot}6 \times 10^8$	$2{\cdot}0 \times 10^8$

Growth of a Yellow-pigmented Gram-negative rod with Acinetobacter 4 in aerated flasks of Synthetic Sewage medium

Experiment 2

Time (h)	Non pigmented Viable Count/ml	Mean	Pigmented Viable Count/ml	Mean
0	$2{\cdot}8 \times 10^5$ $2{\cdot}2 \times 10^5$ $2{\cdot}2 \times 10^5$	$2{\cdot}4 \times 10^5$	$1{\cdot}6 \times 10^4$ $1{\cdot}6 \times 10^4$ $1{\cdot}4 \times 10^4$	$1{\cdot}5 \times 10^4$
6	$5{\cdot}4 \times 10^6$ $4{\cdot}6 \times 10^6$ $4{\cdot}2 \times 10^6$	$4{\cdot}7 \times 10^6$	$4{\cdot}8 \times 10^4$ $4{\cdot}8 \times 10^4$ $4{\cdot}8 \times 10^4$	$4{\cdot}8 \times 10^4$
12	$5{\cdot}4 \times 10^8$ $4{\cdot}8 \times 10^8$ $4{\cdot}6 \times 10^8$	$4{\cdot}9 \times 10^8$	$2{\cdot}2 \times 10^8$ $1{\cdot}6 \times 10^8$ $1{\cdot}6 \times 10^8$	$1{\cdot}8 \times 10^8$

Growth of a yellow-pigmented Gram-negative rod with Pseudomonas putida in aerated flasks of Synthetic Sewage medium

Experiment 1

Time (h)	Non-pigmented Viable Count/ml	Mean	Pigmented Viable Count/ml	Mean
0	$2{\cdot}4 \times 10^5$ $2{\cdot}2 \times 10^5$ $2{\cdot}0 \times 10^5$	$2{\cdot}1 \times 10^5$	$2{\cdot}4 \times 10^4$ $2{\cdot}2 \times 10^4$ $2{\cdot}2 \times 10^4$	$2{\cdot}3 \times 10^4$
4·0	$6{\cdot}2 \times 10^5$ $5{\cdot}4 \times 10^5$ $5{\cdot}2 \times 10^5$	$5{\cdot}6 \times 10^5$	$3{\cdot}6 \times 10^4$ $3{\cdot}2 \times 10^4$ $2{\cdot}8 \times 10^4$	$3{\cdot}1 \times 10^4$
6·0	$1{\cdot}1 \times 10^6$ $9{\cdot}8 \times 10^5$ $6{\cdot}4 \times 10^5$	$8{\cdot}9 \times 10^5$	$7{\cdot}4 \times 10^4$ $7{\cdot}0 \times 10^4$ $7{\cdot}2 \times 10^4$	$7{\cdot}0 \times 10^4$
8·0	$4{\cdot}2 \times 10^6$ $4{\cdot}5 \times 10^6$ $4{\cdot}0 \times 10^6$	$4{\cdot}45 \times 10^6$	$7{\cdot}2 \times 10^5$ $6{\cdot}8 \times 10^5$ $5{\cdot}8 \times 10^5$	$6{\cdot}6 \times 10^5$
10·0	$6{\cdot}0 \times 10^7$ $5{\cdot}6 \times 10^7$ $5{\cdot}2 \times 10^7$	$5{\cdot}6 \times 10^7$	$7{\cdot}8 \times 10^7$ $7{\cdot}6 \times 10^7$ $6{\cdot}0 \times 10^7$	$7{\cdot}1 \times 10^7$
11·0	$6{\cdot}4 \times 10^7$ $5{\cdot}8 \times 10^7$ $5{\cdot}8 \times 10^7$	$6{\cdot}0 \times 10^7$	$2{\cdot}2 \times 10^8$ $1{\cdot}6 \times 10^8$ $1{\cdot}4 \times 10^8$	$1{\cdot}7 \times 10^8$
12·0	$5{\cdot}8 \times 10^7$ $5{\cdot}2 \times 10^7$ $5{\cdot}0 \times 10^7$	$5{\cdot}25 \times 10^7$	$1{\cdot}2 \times 10^8$ $1{\cdot}8 \times 10^8$ $1{\cdot}0 \times 10^8$	$1{\cdot}6 \times 10^8$
16·0	$1{\cdot}4 \times 10^7$ $1{\cdot}2 \times 10^7$ $1{\cdot}2 \times 10^7$	$1{\cdot}25 \times 10^7$	$1{\cdot}1 \times 10^8$ $1{\cdot}1 \times 10^8$ $8{\cdot}0 \times 10^7$	$1{\cdot}0 \times 10^8$

Growth of a yellow-pigmented Gram-negative rod with Pseudomonas putida in aerated flasks of Synthetic Sewage medium

Experiment 2

Time (h)	Non-pigmented Viable Count/ml	Mean	Pigmented Viable Count/ml	Mean
0	$2{\cdot}8 \times 10^5$ $2{\cdot}6 \times 10^5$ $2{\cdot}2 \times 10^5$	$2{\cdot}5 \times 10^5$	$3{\cdot}2 \times 10^4$ $2{\cdot}6 \times 10^4$ $2{\cdot}6 \times 10^4$	$2{\cdot}8 \times 10^4$
4·0	$6{\cdot}2 \times 10^5$ $6{\cdot}0 \times 10^5$ $5{\cdot}2 \times 10^5$	$5{\cdot}8 \times 10^5$	$3{\cdot}2 \times 10^4$ $3{\cdot}2 \times 10^4$ $3{\cdot}2 \times 10^4$	$3{\cdot}2 \times 10^4$
8·0	$5{\cdot}2 \times 10^6$ $5{\cdot}0 \times 10^6$ $4{\cdot}0 \times 10^6$	$4{\cdot}7 \times 10^6$	$7{\cdot}2 \times 10^5$ $6{\cdot}8 \times 10^5$ $6{\cdot}8 \times 10^5$	$6{\cdot}9 \times 10^5$
10·0	$7{\cdot}6 \times 10^7$ $5{\cdot}8 \times 10^7$ $5{\cdot}0 \times 10^7$	$6{\cdot}1 \times 10^7$	$7{\cdot}4 \times 10^7$ $7{\cdot}2 \times 10^7$ $7{\cdot}0 \times 10^7$	$7{\cdot}2 \times 10^7$

APPENDIX 5

Removal of COD, protein and carbohydrate from Synthetic Sewage medium by Acinetobacter 4 and Pseudomonas putida

Removal of COD, protein and carbohydrate from Synthetic Sewage medium by Acinetobacter 4

Experiment 1

Time (h)	Organisms (µgN/ml)	COD (mg/l)	Protein (µg/ml)		Carbohydrate (µg/ml)	
			Exptl	Mean	Exptl	Mean
0		350	262·7	362·1	86·6	85·0
			361·5		83·4	
2	1·1	300	253·5	253·4	72·3	71·6
			253·3		70·9	
4	2·1	248	211·0	210·8	68·1	68·0
			210·6		67·9	
8	3·9	213	192·2	191·9	67·9	66·5
			191·6		65·1	
12	5·1	208	170·1	170·1	66·8	66·3
			170·1		65·8	
16	4·9	210	172·2	172·0	69·0	69·0
			171·8		69·0	

Removal of COD, protein and carbohydrate from Synthetic Sewage medium by Acinetobacter 4

Experiment 2

Time (h)	Organisms (µgN/ml)	COD (mg/l)	Protein (µg/ml)		Carbohydrate (µg/ml)	
			Exptl	Mean	Exptl	Mean
0		374	375·7	375·3	94·6	91·2
			374·9		87·8	
2	1·2	320	262·1	262·1	80·1	80·1
			262·1		80·0	
4	2·25	284	212·7	212·4	79·2	78·4
			212·1		77·6	
6	3·3	224	198·8	198·7	73·3	73·2
			198·6		73·1	
10	5·2	226	181·7	181·2	74·5	73·8
			180·7		73·2	
16	5·1	224	180·0	179·7	73·8	73·6
			179·4		73·4	

Removal of COD, protein and carbohydrate from Synthetic Sewage medium by Acinetobacter 4

Experiment 3

Time (h)	Organisms (µgN/ml)	COD(mg/l)	Protein (µg/ml)		Carbohydrate (µg/ml)	
			Exptl	Mean	Exptl	Mean
0		364	364·0	364·0	85·0	84·1
			364·0		83·25	
4	2·2	256	209·2	208·9	72·0	69·9
			208·6		67·8	
8	4·0	230	190·2	190·1	68·3	68·0
			190·0		67·8	
16	5·3	218	178·8	178·7	68·5	68·0
			178·6		67·5	

Removal of COD, protein and carbohydrate from Synthetic Sewage medium by Acinetobacter 4

Experiment 4

Time (h)	Organisms (µgN/ml)	COD (mg/l)	Protein (µg/ml)		Carbohydrate (µg/ml)	
			Exptl	Mean	Exptl	Mean
0		348	355·1	355·0	91·4	91·2
			354·9		91·0	
10	5·2	207	166·9	166·8	72·1	71·3
			166·7		70·5	

Removal of COD, protein and carbohydrate from Synthetic Sewage medium by Pseudomonas putida

Experiment 1

Time (h)	Organisms (µgN/ml)	COD (mg/l)	Protein (µg/ml)		Carbohydrate (µg/ml)	
			Exptl	Mean	Exptl	Mean
0		350	362·4	362·2	87·5	87·2
			362·0		86·9	
2	1·0	316	307·9	307·7	69·9	69·8
			307·5		69·7	
4	2·1	379	264·6	264·3	66·8	66·2
			264·0		65·6	
5	3·85	255	231·8	231·7	69·9	61·9
			231·6		61·9	
6	6·6	227	203·1	202·7	59·2	58·3
			202·3		57·5	
8	8·8	214	188·2	188·2	57·7	57·5
			188·2		57·3	
10	10·5	204	184·9	184·6	56·0	55·75
			184·3		55·5	
12	10·0	216	190·1	190·1	56·3	56·1
			190·1		55·9	
16	9·2	240	198·7	198·5	56·7	56·5
			198·3		56·4	

Removal of COD, protein and carbohydrate from Synthetic Sewage medium by Pseudomonas putida

Experiment 2

Time (h)	Organisms (µgN/ml)	COD (mg/l)	Protein (µg/ml)		Carbohydrate (µg/ml)	
			Exptl	Mean	Exptl	Mean
0		374	375·3	375·3	92·1	91·7
			375·3		91·3	
2	1·2	336	315·9	315·6	72·9	72·2
			3·5·3		71·6	
8	9·1	232	203·7	203·3	58·3	58·0
			202·9		57·7	
10	11·0	224	198·9	198·8	55·8	55·8
			198·7		55·8	
12	10·8	230	198·9	198·6	56·5	56·4
			198·3		56·3	
16	8·9	236	200·2	57·0	56·6	
			200·2		56·3	

Removal of COD, protein and carbohydrate from Synthetic Sewage medium by Pseudomonas putida

Experiment 3

Time (h)	Organisms (µgN/ml)	COD (mg/l)	Protein (µg/ml		Carbohydrate (µg/ml)	
			Exptl	Mean	Exptl	Mean
0		348	355·8	355·7	83·6	82·8
			355·6		82·0	
10	10·8	202	181·5	181·4	53·8	52·9
			181·3		52·0	

APPENDIX 6

Removal of COD, protein and carbohydrate from culture filtrates by Acinetobacter 4 and Pseudomonas putida

Removal of COD, protein and carbohydrate by Acinetobacter 4

from its own culture filtrate

Time (h)	Organisms (µgN/ml)	COD (mg/l)	Protein (µg/ml)		Carbohydrate (µg/ml)	
			Exptl	Mean	Exptl	Mean
0		207	166·9	166·8	72·1	71·3
			166·7		70·5	
2·0	1·1	183	138·5	138·5	70·5	69·5
			138·5		68·5	
6·0	2·0	167	124·5	124·2	70·6	70·4
			123·9		70·2	
10·0	4·0	143	113·6	113·6	70·8	70·8
			113·6		70·8	
12·0	5·0	139	99·8	99·4	70·7	70·5
			99·0		70·3	
16·0	4·8	143	105·3	105·2	70·2	69·8
			105·1		69·4	

Removal of COD, protein and carbohydrate by Acinetobacter 4 from culture filtrates from Pseudomonas putida

Time (h)	Organisms (µgN/ml)	COD (mg/l)	Protein (µg/ml)		Carbohydrate (µg/ml)	
			Exptl	Mean	Exptl	Mean
0	1	202	181·4	181·4	53·8	52·9
			181·4		52·0	
2·0	1·2	185	156·9	156·6	53·9	53·7
			156·3		53·5	
6·0	2·0	167	138·9	138·8	52·4	52·0
			138·7		51·6	
10·0	4·0	145	119·6	119·3	52·7	52·6
			119·0		52·5	
12·0	5·1	143	110·5	110·4	52·8	52·3
			110·3		51·8	
16·0	4·8	144	112·4	112·3	52·5	52·4
			112·2		52·3	

Removal of COD, protein and carbohydrate by Pseudomonas putida from its own culture filtrate

Time (h)	Organisms (µgN/ml)	COD (mg/l)	Protein (µg/ml)		Carbohydrate (µg/ml)	
			Exptl	Mean	Exptl	Mean
0	1	202	181·5	181·4	53·8	52·9
			181·3		52·0	
2·0	1·0	192	160·5	160·2	47·3	47·1
			159·9		46·9	
4·0	2·0	186	149·5	149·5	45·1	44·8
			149·5		44·5	
6·0	3·85	167	135·7	135·3	43·9	43·5
			134·9		43·1	
8·0	6·8	165	126·8	126·8	41·7	41·6
			126·8		41·5	
10·0	9·0	139	117·5	117·5	39·8	39·7
			117·5		39·6	
12·0	8·7	142	121·4	121·2	40·5	40·3
			121·0		40·1	
16·0	8·5	148	128·7	128·4	40·2	40·2
			128·1		40·2	

Removal of COD, protein and carbohydrate by Pseudomonas putida from culture filtrate from Acinetobacter 4

Time (h)	Organisms (µgN/ml)	COD (mg/l)	Protein (µg/ml)		Carbohydrate (µg/ml)	
			Exptl	Mean	Exptl	Mean
0	1	207	171·1	166·8	72·1	71·3
			166·5		70·5	
2·0	1	197	149·2	149·1	63·0	62·9
			149·0		62·8	
4·0	2·1	184	142·0	142·0	58·2	57·9
			142·0		57·6	
6·0	3·75	165	127·9	127·8	55·7	55·6
			127·7		55·5	
8·0	6·6	148	117·5	117·2	52·0	51·9
			116·9		51·8	
10·0	9·2	139	110·1	110·0	49·3	48·8
			109·9		48·3	
12·0	8·7	143	115·6	115·4	50·0	49·7
			115·2		49·4	
16·0	8·2	141	120·4	120·2	48·8	48·6
			120·0		48·4	

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